

THE NEUROENDOCRINOLOGY OF OVINE PARTURITION:
EFFECT OF ESTROGENS AND ANDROGENS ON FETAL ACTH SECRETION
IN LATE GESTATION

By

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To my friends: thank you for standing beside me.

To my family: thank you for believing in me.

To my husband: thank you for loving me.

In memory of my Grandfather. I wish you could have been here.

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KEY TO ABBREVIATIONS

ACTH.....	adrenocorticotrophic hormone
ADH.....	anti-diuretic hormone
ANOVA.....	analysis of variance
ANP.....	atrial natriuretic peptide
Arg.....	arginine
Asn.....	asparagine
AVP.....	arginine vasopressin
B/I.....	bioactive to immunoreactive ratio
BID.....	twice a day
BSA.....	bovine serum albumin
cAMP.....	cyclic adenosine monophosphate
cDNA.....	complementary deoxyribonucleic acid
CRF.....	corticotropin releasing factor
Cys.....	cysteine
dex.....	dexamethasone
DNA.....	deoxyribonucleic acid
F.....	cortisol
Gln.....	glutamine

Gly.....	glycine
GRE.....	glucocorticoid response element
HCl.....	hydrochloric acid
HPA.....	hypothalamus-pituitary-adrenal
HPD.....	hypothalamus-pituitary disconnection
HSP.....	heat shock protein
HSS.....	hydrocortisone sodium succinate
Ile.....	isoleucine
iACTH.....	immunoreactive ACTH
iAVP.....	immunoreactive arginine vasopressin
iCRF.....	immunoreactive corticotropin releasing factor
ID.....	inner diameter
Ig.....	immunoglobulin
IM.....	intramuscular
IV.....	intravenous
kD.....	kilodalton
Leu.....	leucine
M.....	molar
MAP.....	mean arterial pressure
mmHg.....	millimeters of mercury
N.....	normal
NiPr.....	nitroprusside
NO.....	nitric oxide

NPY.....	neuropeptide Y
NTS.....	nucleus of the tractus solitarius
oCRF.....	ovine corticotropin releasing factor
OD.....	outer diameter
O.D.	optical density
ovx.....	ovariectomized
P _a CO ₂	arterial partial pressure of carbon dioxide
P _a O ₂	arterial partial pressure of oxygen
Phe.....	phenylalanine
POMC.....	proopiomelanocortin
Pro.....	proline
PVN.....	paraventricular nucleus
RNA.....	ribonucleic acid
RIA.....	radioimmunoassay
SON.....	supraoptic nucleus
Tyr.....	tyrosine

Abstract of Dissertation Presented to the Graduate School
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Parturition in the sheep is controlled by fetus. Near the end of gestation, plasma adrenocorticotrophic hormone (ACTH) and cortisol concentrations are increased, stimulating estrogen production from the placenta which acts on the uterus to stimulate contractions leading to labor and delivery. It has previously reported that cortisol negative feedback efficacy is decreased at the end of gestation. We hypothesized that increases in fetal plasma androgen and estrogen concentrations in the fetus might increase plasma ACTH concentrations, either by stimulating ACTH secretion or by altering the negative feedback effect of cortisol on ACTH. Fetal sheep were chronically catheterized and implanted with 17 β -estradiol and/or androstenedione pellets (approximately 250 μ g per day) between 110 and 125 days gestation. Fetal plasma ACTH and cortisol

concentrations were measured throughout the duration of the pregnancy and ACTH responses to hypotension and the effectiveness of cortisol inhibition of ACTH secretion were tested. The effect of estradiol and androstenedione were independent of each other and significant. Estradiol treatment increased basal plasma ACTH concentrations as well as stress-induced ACTH secretion (approximately 2-fold). Estradiol, however, did not have a significant effect on cortisol negative feedback sensitivity. Androstenedione significantly decreased the ability of cortisol to inhibit plasma ACTH secretion without influencing basal or stimulated plasma ACTH concentrations. When estradiol and androstenedione were administered together, the combined effect was to increase plasma ACTH and cortisol concentrations prematurely and significantly decrease the length of gestation when compared to control fetuses (141.7 and 145.8, respectively). In summary, increases in exogenous 17β -estradiol concentrations augmented fetal plasma ACTH secretion. Increases in exogenous androstenedione concentrations decreased cortisol negative feedback efficacy of plasma ACTH secretion. When these two gonadal steroids were given in combination, the mean gestation length was significantly decreased. These results suggest that androgens and estrogens may play a role in stimulating fetal plasma ACTH secretion and therefore contribute to the initiation of parturition in the sheep.

CHAPTER 1 INTRODUCTION

Parturition in the sheep is stimulated by activation of the fetal hypothalamus-pituitary-adrenal (HPA) axis (Challis and Brooks, 1989; Liggins et al., 1973). At approximately 85% gestation, drive to the fetal HPA is stimulated and fetal plasma adrenocorticotrophic hormone (ACTH) and cortisol concentrations begin to increase. At approximately 95% gestation, there is a further increase in plasma ACTH and cortisol secretion due to a decrease in cortisol negative feedback efficacy (Wood, 1988). Cortisol acts at the placenta to increase cytochrome P450_{c17} (17 α -hydroxylase and 17,20 lyase activities) which increases production of nineteen and eighteen carbon steroids from progesterone. These steroids are subsequently converted to estrogens and androgens, increasing fetal plasma concentrations of both. With this conversion, there is a shift in the plasma ratio of progesterone to estrogen. Estrogen promotes uterine contractions by the formation of gap junctions (see Chap. 2)(Garfield 1977, 1984; Garfield et al., 1978), as well as direct and indirect actions on the uterus itself along with prostaglandins and oxytocin (Lye et al., 1983a; Fuchs et al., 1982), promoting the initiation of labor.

In the sheep, the HPA axis must be intact for parturition to occur normally. Destruction or inhibition of any part of the axis leads to an abnormal length of gestation. In a review by Nathanielsz et al., it was stated that it is "necessary to look for a stimulus to the fetal hypothalamus which is not present at an earlier stage of gestation and is removed

by parturition" (1972, p. 40). This is the key to determining what drives parturition in all species, including the sheep. The hypothesis of these studies is that the maturation of the fetal HPA axis involves gonadal steroids produced by the placenta. Increasing fetal plasma cortisol concentrations then act on the placenta to induce cytochrome P450 enzyme action, shifting placental steroid production from progesterone to androgens and estrogens. These gonadal steroids then can act on the fetal brain to augment plasma ACTH secretion and possibly facilitate parturition.

To test this hypothesis, the effects of estradiol and androstenedione on the fetal hypothalamus-pituitary-adrenal axis function were examined. The normal developmental pattern of plasma ACTH, cortisol, estrogen and androgen secretion was examined as well as the tissue content of releasing factors for ACTH and the processing of ACTH in the pituitary. Glucocorticoid receptor protein in the fetal hypothalamus and pituitary was examined as well. Finally, the combined effects of estrogen and androgen on the timing of parturition were examined. With these studies, we believe that the role of estrogens and androgens in the control of parturition in this species will be determined.

CHAPTER 2 REVIEW OF THE LITERATURE

2.1 Control of Parturition

After numerous observations and experiments, in 1973, Liggins et al. concluded that parturition in the sheep is controlled by the fetus. This is accomplished by the coordination of many maturational processes and endocrine factors, working in concert, which culminate in the expulsion of a healthy, fully developed fetus. This process is intricately linked to the hypothalamus-pituitary-adrenal axis in the fetus. Prior to the pioneering studies by Liggins, a natural phenomenon in sheep was reported (Binns et al., 1964). If ewes ate a particular plant (*Veratrum californicum*) on day 14 of gestation, the birth of the fetus was delayed indefinitely until caesarian section or death of the fetus. The fetuses had a number of birth defects, particularly cyclopia and a dysfunctional hypothalamus-pituitary (HP) axis due to dislocation of the pituitary from its normal position. The connection between the malformed, dysfunctional HP axis and the delayed parturition would soon be realized as a significant event.

2.1.1. Ontogeny and Maturation within the Hypothalamus-Pituitary-Adrenal Axis

Development of the ovine fetal hypothalamus-pituitary-adrenal axis begins during the first third of gestation (term being 145-148 days) with the formation of the pituitary and adrenal glands. The fetal pituitary gland can be detected as early as 31 days gestation and differentiation of the anterior pituitary seen at about 40 days (Perry et al., 1982).

Staining of cells in the anterior pituitary indicate the presence of ACTH immunoreactivity by as early as 50 days gestation and by 60 days gestation, processing of ACTH from proopiomelanocortin (POMC) can be detected in the intermediate lobe of the pituitary in the fetal sheep (Mulvogue et al., 1986).

The fetal adrenal can be identified by approximately 28 days gestation in the sheep (Wintour et al., 1977). By 40-50 days gestation, in vivo experiments demonstrate that the fetal adrenal readily secretes cortisol in response to ACTH (Wintour et al., 1975; Glickman and Challis, 1980). Between 90 and 120 days gestation, the cells of the zona fasciculata (which are responsible for the synthesis of cortisol) are relatively immature (Robinson et al., 1979) and approximately 90% of fetal plasma cortisol is derived from the maternal circulation (Hennessy et al., 1982). After 120 days gestation, fetal adrenal sensitivity to plasma ACTH increases (Liggins et al., 1973; Rose et al., 1982), the proportion of cortisol that is of fetal origin increases (Hennessy et al., 1982a), and the correlation between fetal ACTH secretion and fetal cortisol secretion becomes significant (Hennessy et al., 1982b). Adrenal weight also increases as a function of gestational age (Comline and Silver, 1961).

Early studies involving fetal hypophysectomy (Liggins et al., 1967) and adrenalectomy (Drost and Holm, 1968) with subsequent prolongation of pregnancy suggested a link between adrenal steroid production and parturition. If this is the case, fetal plasma cortisol secretion should be altered as gestation nears an end. Before about 130 days gestation, fetal corticosterone levels are low, but several days before parturition, plasma levels increase and peak at birth (in the chronically instrumented animal; Bassett and Thorburn, 1969). Nathanielsz et al. (1972) found that fetal plasma cortisol

concentrations began to increase about 3-4 days before parturition and then steadily declined in the newborn lamb. A more elaborate study of cortisol secretion was performed by Magyar et al. (1980) in which exponential curves were fit to the data to more accurately describe the increase in fetal plasma cortisol concentrations. This analysis revealed fetal plasma cortisol concentrations increasing exponentially about 10-15 days prior to parturition. Cortisol is secreted in response to ACTH binding to receptors at the adrenal gland. In vivo experiments by Brown et al. (1978) demonstrated that not only did glucocorticoids increase with development, but the ability of the fetal adrenal to secrete cortisol in response to ACTH was observed around 120-129 days gestation. The increase in cortisol secretion that occurs near the end of gestation is in part due to an increase in adrenal sensitivity to stimulation by ACTH (Madill and Bassett, 1973) but also possibly due to increased plasma ACTH secretion from the fetus.

Jones et al. (1977) presented some of the first data examining the changes in fetal plasma ACTH concentrations during development. They demonstrated that fetal plasma ACTH concentrations increase prior to parturition. However, they concluded that the increase occurred after the increase in fetal plasma cortisol and therefore is probably not the reason for the changes in fetal plasma cortisol concentrations. Experiments performed later by other investigators (accounting for episodic secretion of ACTH, stress-induced ACTH secretion, and cortisol negative-feedback inhibition of ACTH secretion) revealed that fetal plasma ACTH concentrations increase much sooner than previously described by Jones et al. (MacIsaac et al., 1985; Wintour, 1984; Norman et al., 1985). In fact, fetal plasma ACTH concentrations increase during the last 30 days of gestation, well before the increase in plasma cortisol concentrations which occur at approximately 120 days gestation.

The results of increasing plasma ACTH concentrations in vivo when taken together with the in vitro pituitary ACTH secretion studies support the presence of an additional factor involved in the process of parturition. Basal output of ACTH from fetal sheep pituitaries in culture does not increase as a function of gestational age (Durand et al, 1986). Pituitary ACTH secretion in culture does not increase during the last week of gestation at a time when in vivo circulating plasma ACTH concentrations are increasing exponentially (McMillen and Merei, 1993). These data suggest that the preparturient increase in ACTH secretion is dependent on some other factor (hypothalamic or other) to stimulate secretion and not a function of basal pituitary output. In addition, McMillen and Merei (1993) also found no change in responsiveness of the fetal corticotroph to CRF as a function of gestational age. However, prior exposure of the fetal pituitary to cortisol increased its responsiveness to CRF. There appears to be a signal, possibly from the hypothalamus, that increases the activity of the fetal pituitary and therefore the fetal adrenal and is the trigger to parturition in the sheep.

2.1.2 Induction of Parturition by Stimulation of the HPA axis

The involvement of the fetal adrenal cortex in the initiation of parturition in the sheep was suggested after in utero plasma concentrations of corticosteroids revealed dramatic increases in these hormones prior to birth (Bassett and Thorburn, 1969). Before this conclusion was made, a number of studies aimed at investigating the role of the pituitary-adrenal axis in the birth process were performed in fetal sheep by Liggins (1968). ACTH infused (0.1 mg/24hrs) into fetal sheep induced parturition within 4-7 days, along with producing adrenal hypertrophy. Cortisol infusions into the fetus induced parturition within 5 days. The same doses of ACTH or cortisol infused into the ewes did not induce parturition. Although 17β -estradiol at 2mg/24hr had no effect, an infusion of cortisol at

25mg/24 hr plus the estradiol resulted in spontaneous delivery after about 4 days. (Liggins, 1968).

Further studies by Liggins (1969) showed that it was glucocorticoid activity (and not mineralocorticoid activity) that was important for initiation of parturition. Evidence for this effect was shown by the inability of deoxycorticosterone or corticosterone to induce parturition. Dexamethasone infused at rates of 0.06-4 mg/24hr in the fetus and 4mg/24hr in the ewe was ineffective in producing premature delivery.

Since the earlier experiments of Liggins, many other studies have been performed which confirm the previous results. Parturition was induced by continuous IV cortisol infusion at 130 days gestation (increasing the infusion rate from 2.8mg/24hours up to 44.8mg/24hr over 3 days) (Thomas et al., 1978). Activation of fetal adrenal function by pulsatile ACTH administration in 125-127 day fetal sheep induced labor and delivery in 4-5 days, resulting in 4-6 fold elevation in fetal plasma cortisol concentrations (Lye et al., 1983). Hypophysectomized fetuses administered Dexamethasone or ACTH infusions exogenously undergo parturition (Kendall et al., 1977).

2.1.3 Disruption of the HPA Axis Delays Parturition

Parturition can be induced artificially by increasing the activity of the fetal HPA (as described above) or artificially delayed by blocking the normal process at one of the three levels; at the hypothalamus, pituitary, or the adrenal gland. Adrenalectomy in the late gestation fetal sheep delays parturition (Drost and Holm, 1968). Pituitary ablation also inhibits the initiation of parturition (Liggins et al., 1967). Disconnection of the hypothalamus from the pituitary (HPD) in fetal sheep between 108-112 days gestation delayed birth by at least 8 days (Antolovich et al., 1990). Following hypophysectomy,

infusions of ACTH or glucocorticoid still induce parturition (Kendall et al., 1977). Further studies from this group also demonstrated the necessity of the HP axis in maturation of pituitary corticotrophs (Kendall et al., 1977). After HPD, fetuses were infused with saline or cortisol. In the cortisol-infused fetuses, the proportion of fetal-type corticotrophs was significantly lower than in the saline-infused fetuses however the number of adult-type corticotrophs did not change. A direct effect of cortisol on pituitary corticotroph maturation was seen but complete maturation required the presence of a complete hypothalamus-pituitary axis (Antolovich, et al., 1992). More specifically, following destruction of the fetal paraventricular nuclei of the hypothalamus, parturition was delayed (McDonald and Nathanielsz, 1991). Therefore, the signal for parturition may either be sent to the PVN (receives input from the NTS, amygdala and/or hippocampus) or possibly be derived in the CRF and AVP producing neurons of the PVN.

2.1.4 Alterations in Fetal Gonadal Steroids in Late Gestation

In addition to the increase in fetal plasma ACTH and cortisol concentrations that occur at the end of gestation, fetal plasma estrogen and androgen concentrations also increase. Plasma estrone concentrations appear to increase over the last four days of gestation while estradiol may be increasing over the last 8 days in the fetus and amniotic fluid (Challis and Patrick, 1981) (Findlay and Cox, 1970). In a paper by Challis and Patrick, fetal plasma estrone concentrations increased from 40 pg/ml at 14 days prior to birth to near 400 pg/ml the day before birth. Fetal plasma estradiol concentrations increased from 20 pg/ml at -14 days to 80 pg/ml on the day before parturition. Plasma estradiol concentrations in the ewe increased over the last 2 days of pregnancy in the ewe (Robertson and Smeaton, 1973), with a 10-fold increase on the day before parturition,

from 20–40 pg/ml up to 411 pg/ml (Challis, 1970). At the time plasma estrogens increase, there was a decrease in plasma progesterone (Bedford et al., 1972). This is possibly due to the conversion of estrogen from progesterone, with plasma progesterone acting as sink for plasma estrogen production. Since progesterone is also a precursor for androgen production, one would expect to see increases in plasma concentrations of these steroids as well. Plasma androstenedione and testosterone concentrations do indeed increase in the late gestation fetus in a manner similar to estrogen concentrations (Pomerantz and Nalbandov, 1975; Yu et al., 1983).

This increase in plasma androgen and estrogen concentrations is a result of the actions of cortisol on the placenta (Anderson et al., 1975; Steele et al., 1976). Although the placenta has aromatase activity, the plasma concentrations of androgens and estrogens are very low throughout gestation (Mann et al., 1975). Cortisol acts at the placenta to induce an enzyme, cytochrome P450_{C17}, which has 17 α -hydroxylase and 17,20 lyase activities. Upon induction, P450_{C17} facilitates the production of estrogens and androgens from progesterone, thus increasing plasma concentrations of these hormones.

Gap junctions in the myometrium, which are very important for synchronized uterine contractions are believed to be formed at the time of progesterone withdrawal (Garfield et al., 1977). It has been proposed that as a result of decreased progesterone and increased estrogen, there is an augmentation in synthesis of proteins associated with gap junctions (Garfield et al., 1978; Garfield, 1984, 1977). These gap junction proteins are inserted into the plasma membrane of the myometrium and aggregate to form gap junctions. Gap junctions are necessary for the uterus to contract in a unified, coordinated fashion in order to expel the fetus. Prostaglandins and oxytocin, as well as estradiol (Lye et al., 1983) act in concert to stimulate uterine contractions in the process of labor.

Tissue estrogen concentrations in sheep also increase towards term or after ACTH induced labor, especially in the myometrium (Power and Challis, 1987). The ovine placenta, through sulfatase and aromatase activities, converts estrone and estrone sulfate to 17 β -estradiol, the more potent estrogen for the target organ, the myometrium (Rossier and Pierrepont, 1974).

2.2 Regulation of ACTH Secretion

Secretion of ACTH is primarily controlled by corticotropin releasing factors of the hypothalamus, namely CRF and AVP. CRF and AVP are synthesized in the parvocellular neurons of the paraventricular nucleus of the hypothalamus and project to the median eminence (Reeves and Andreoli, 1992). These CRFs are secreted into the external zone of the median eminence. The peptides then diffuse from the pericapillary space into the primary capillaries of the hypothalamo-pituitary portal circulation (Leranth et al, 1983). CRFs are carried down the infundibular stalk via long portal vessels to the anterior pituitary, where they are released into the extracellular space surrounding the cells of the anterior pituitary and act on the corticotrophs via their specific receptors to increase ACTH secretion. Corticotrophs produce ACTH through the step-wise processing of pro-opiomelanocorticotropin (POMC) to produce ACTH₁₋₃₉ which is secreted into the general systemic circulation. Upon reaching the adrenal gland, ACTH stimulates adrenal cortical secretion of glucocorticoids. These glucocorticoids then feed back at the hypothalamus and pituitary to inhibit ACTH secretion (Keller-Wood and Dallman, 1984). This is demonstrated pictorially in Figure 2.1.

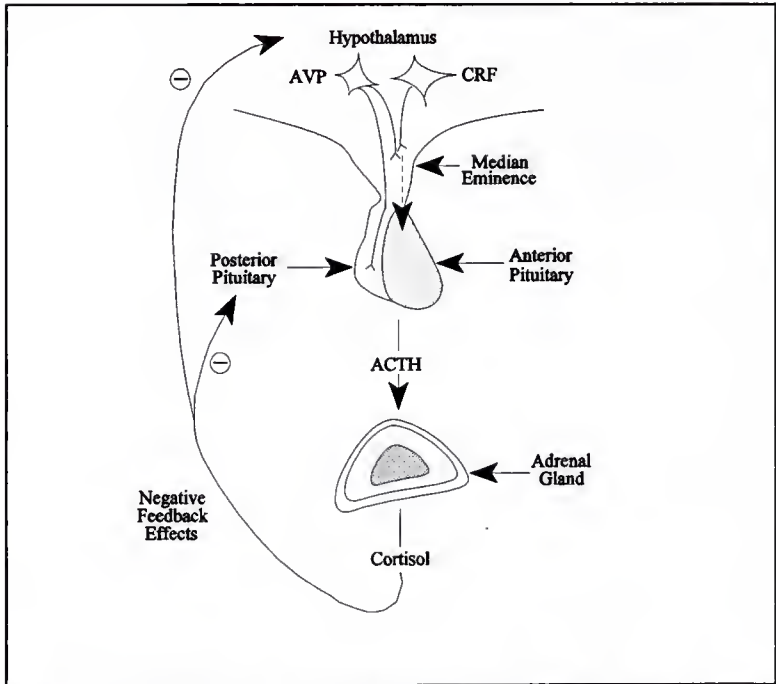


Figure 2.1. Hypothalamic-Pituitary-Adrenal (HPA) Axis. (-) indicates action of cortisol to inhibit ACTH secretion at the pituitary and inhibit AVP and CRF secretion at the hypothalamus.

2.2.1 Corticotropin-Releasing Factor

Since 1955 it has been known that the hypothalamus contained substances that acted at the pituitary gland to increase ACTH secretion in vitro (Guillemin and Rosenberg, 1955; Saffran and Schally, 1955). In 1981, Vale et al. characterized a 41-amino acid peptide from sheep hypothalamus that stimulated ACTH secretion from corticotrophs (Vale et al., 1981) and published the primary structure of ovine CRF (oCRF) (Spiess et al., 1981). oCRF was further characterized when the cDNA was cloned and sequenced

(Furutani, 1983). At this time a similarity was discovered between the precursor proteins for CRF, AVP and ACTH which implies a common evolutionary beginning. A 20 kilodalton immunoreactive form of CRF (from which CRF₁₋₄₁ can be generated by proteolytic cleavage) was identified from rat hypothalamus and is close to the value for ovine and human pre-pro-CRF based on their cDNA sequences (Laubert et al., 1984).

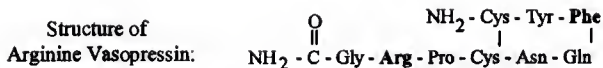
CRF increases ACTH secretion from the anterior pituitary gland by binding to high affinity receptors (Wynn et al., 1983) located on the corticotrophs (Leroux and Pelletier, 1984). Activation of the receptor complex increases adenylate cyclase activity (Perrin et al., 1986) and cAMP, which results in an increase in ACTH secretion. In cultured rat pituitary cells, CRF can enhance the rates of ACTH synthesis as well as release (Vale et al., 1983). ACTH release can be modulated by down-regulation of CRF receptors in the anterior pituitary. There is evidence that CRF (Wynn et al., 1988) AVP (Hauger and Aguilera, 1993) and glucocorticoids (Hauger et al., 1987; Schwartz et al., 1986; Wynn et al., 1985) can all act to regulate CRF receptor number. Therefore, an alteration in CRF receptor number, receptor activity, receptor coupling or even corticotroph number can affect the ability of CRF to stimulate ACTH secretion.

2.2.2 Arginine Vasopressin

The other major regulator of ACTH secretion of hypothalamic origin is arginine vasopressin (AVP). Classically, AVP is known as anti-diuretic hormone (ADH) for its role in renal regulation of fluid balance. An increase in plasma osmolality is the most "potent" stimulus to AVP secretion in that very small increases in osmolality cause an almost immediate secretory response from the posterior pituitary. AVP binds to receptors on the basolateral membrane of the cortical and medullary collecting ducts of the nephron.

Binding to these receptors (V_2) results in activation of adenylate cyclase, which subsequently increases cyclic AMP. This second messenger is then thought to facilitate an increase in protein channels found in the luminal membrane, thereby increasing the diffusion of water out of the nephron and concentrating urine while retaining fluid (Vander, 1985). In addition to AVP's role in modulating fluid reabsorption in the renal system, AVP is also a potent vasoconstrictor of the cardiovascular system (binding to V_1 receptors). Significant decreases in blood volume (15-20%) produce large increases in plasma vasopressin (as much as 20-fold). Decreases in blood volume are sensed as decreased stretch of the arterial baroreceptors located in the carotid sinus as well as receptors in the left atrium and AVP secretion is reflexively stimulated (Berne and Levy, 1986).

AVP is one of the two hormones secreted from the posterior pituitary gland (oxytocin, being the other). The posterior pituitary, also called the neurohypophysis, is comprised of axons and axon terminals, which account for 42% of its total volume (Nordmann, 1977). These axons project from magnocellular neurons of the supraoptic nucleus (SON) and paraventricular nucleus (PVN) of the hypothalamus. AVP and oxytocin are structurally similar hormones (differing by only 2 amino acids) with very different functions. AVP has actions on the renal and circulatory systems while oxytocin causes milk ejection and uterine contractions. AVP and oxytocin are produced in different neurons of the same nuclei and are stored in secretory vesicles or granules with their appropriate neurophysin (Silverman and Zimmerman, 1975).



Structure of oxytocin: same as for AVP except that **Leu** replaces **Arg** and **Ile** replaces **Phe**.

AVP is produced from a large precursor protein containing not only AVP, but also neurophysin and a glycopeptide, "signal" sequence (Sachs et al., 1969). The proposed structure of this molecule is shown in Fig. 2.2, reproduced from Gainer et al. (1980).

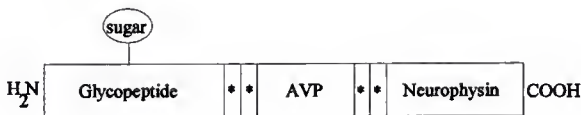


Figure 2.2. Proposed structure of the precursor protein for vasopressin. A sugar moiety is found attached to the glycopeptide, or signal sequence. The (*) represent basic amino acid residues, which serve as cleavage sites in the precursor molecule.

The precursor protein molecule is packaged in granules with the enzymes needed for processing AVP to its final form. As the granules move down the axons, posttranslational processing of the precursor molecule occurs within the granules. When the granules reach the axon terminals, the nerve is depolarized and the granules are exocytosed, the contents of the granules (AVP hormone, neurophysin, and fragments of precursor) are released (Brownstein et al., 1980). Magnocellular neurons containing AVP project fibers to the

median eminence (external and internal zones) and therefore may be important in the regulation of ACTH secretion (Holmes et al., 1986).

AVP is a potent modulator of pituitary ACTH secretion. In fact in sheep, AVP is a more potent stimulator of ACTH secretion than CRF (Familiari et al., 1989; Liu et al., 1990). AVP binds to receptors on the anterior pituitary corticotroph to increase plasma ACTH secretion. These receptors are different from the pressor receptors (subtype V_1) or antidiuretic (subtype V_2) that are found in the periphery. Data from two different groups (Baertschi and Friedli, 1985; Jard et al., 1986) suggest that a subtype (classified as V_{1b} or V_3), distinct from the peripheral receptors, exists in the brain with protein kinase C as its second messenger.

AVP can effect anterior pituitary secretion in two ways; either by AVP secretion from axons which terminate in the median eminence or by AVP secretion from the posterior pituitary. Evidence for both possibilities exists. AVP containing neurons of the SON and PVN are known to project to the external zone of the median eminence (Hoffmann et al., 1989). A further distinction has been made in that AVP-containing parvocellular neurons of the PVN were found to innervate the external zone of the median eminence. The magnocellular neurons of the PVN pass through the internal zone of the median eminence, to the neurohypophysis, but also contribute to the AVP found in the median eminence (Holmes et al., 1986). AVP secretion from the posterior pituitary gland has also been implicated in plasma ACTH secretion. In studies performed in dogs, neurohypophysectomy attenuated the plasma ACTH response to hypotension. After restoration of plasma AVP levels to those observed in the intact animal, plasma ACTH levels were almost completely returned to normal (Raff et al., 1988). The action of AVP appears to be through a direct effect at the pituitary gland.

2.2.3 CRF and AVP as Regulators of ACTH Secretion

The synergistic activity of CRF and AVP on ACTH secretion has been well documented. In support of this is an overwhelming amount of anatomical data for the interaction between these two secretagogues. CRF and AVP are found in the same neurosecretory vesicles in the median eminence (Whitnall et al., 1985). In normal rats, staining of the median eminence by immunocytochemistry revealed co-localization of AVP in 50% of CRF axons (Whitnall et al., 1987). Repeated stress (immobilization in rats) increases the co-localization of AVP in CRF nerve terminals in the median eminence (de Goeij et al., 1991). Following adrenalectomy in rats, CRF immunostaining increases in parvocellular neurons of the PVN and the amount of co-localization with AVP increases (Sawchenko et al., 1984). The increase in CRF and AVP immunoreactivity following adrenalectomy is prevented by intracerebral injection of dexamethasone (Sawchenko, 1987).

From in vitro studies performed in cultured pituitary cells, CRF is considered to be the more potent secretagogue for ACTH secretion in rats while AVP appears to be a more potent stimulus to ACTH release in sheep (Familarì et al., 1989). However, the action of each is potentiated when administered together in cultured pituitary cells (Gillies et al., 1982), adult freely moving rats (Rivier and Vale, 1983b) and in fetal sheep (Brooks and White, 1990). In a study by Engler et al. (1989) performed in conscious sheep, stress-induced ACTH secretion (audiovisual and insulin-induced hypoglycemia) was accompanied by increases in hypothalamic CRF and AVP secretion. What is most interesting is that the CRF:AVP molar ratio was altered with the stress. Portal plasma AVP was increased above that of CRF, increasing the ratio of AVP to CRF. Since CRF

and AVP can be found in the same neurosecretory vesicles, this suggests that differential regulation of each individual hormone also occurs. A synergistic effect of CRF and AVP on ACTH secretion was also observed as seen by a lack of 1:1 concordance between hypothalamic AVP/CRF secretion and pituitary ACTH secretion. Engler et al. suggested that this effect may be due to secretion of other hypothalamic factors which increased ACTH secretion.

2.2.4 Other ACTH Secretagogues

The control of adrenocorticotropin secretion is a complex process involving numerous factors (neurotransmitters and neuropeptides) that augment ACTH secretion. Rat pituitary corticotrophs in culture release ACTH in response to epinephrine and norepinephrine acting on α_1 -adrenergic receptors (Giguere et al., 1981). Epinephrine has also been identified in portal plasma suggesting a physiological role in the control of anterior pituitary function (Johnston et al., 1983). Neuropeptide-Y injected centrally in fetal and adult sheep increase plasma ACTH concentrations but does not stimulate the pituitary directly (Brooks et al., 1994). This suggests that NPY acts centrally to increase the activity of the HPA axis. Evidence for NPY stimulation of CRF secretion supports this conclusion (Haas and George, 1987). Endogenous opioids are capable of stimulating the HPA axis in late gestation fetal sheep but do not tonically stimulate the axis in a regulatory manner (Brooks and Challis, 1988). Serotonin stimulates ACTH secretion in humans, demonstrated by pharmacologically increasing serotonin with fenfluramine (Lewis and Sherman, 1984). However, there are conflicting data as to the site of action of serotonin (hypothalamus or pituitary). In rats, CRF secretion is decreased after elimination of endogenous hypothalamic catecholamines suggesting a role for central

catecholinergetic neurons in control of ACTH release (Guillaume et al., 1987). Angiotensin II also increases plasma ACTH secretion by induction of CRF (Rivier and Vale, 1983). Prostaglandin E₂ alone does not increase ACTH secretion but enhances the ability of AVP to stimulate ACTH secretion with no effect on CRF (Brooks and Gibson, 1992). In addition to stimulating activity of the HPA axis, factors from the brain also inhibit the axis. The dopaminergic system in the amygdaloid central nucleus has been found to inhibit ACTH secretion by action on the anterior and lateral hypothalamus (Beaulieu, et al., 1987). Atrial-natriuretic peptide (ANP) has also been shown to alter ACTH secretion. Brain ANP is secreted into the hypophyseal portal vessels from the hypothalamus and physiological concentrations inhibit ACTH release from pituitary cells in vitro (Dayanithi and Antoni, 1989; Lim et al., 1990; Sheward et al., 1991). In vivo immunoneutralization of ANP significantly increased ACTH release but had no effect on release during ether stress (Fink et al., 1991). These results suggest a role for ANP as a mediator in the regulation of ACTH secretion.

2.3 Negative Feedback Regulation of ACTH Secretion by Cortisol

Glucocorticoids are very versatile steroids and necessary for survival in a number of species, including sheep and primates. Binding of the glucocorticoid to its receptor promotes binding to and transcription of DNA, production of mRNAs for synthesis of enzymes, and eventually alteration of cell function. Glucocorticoids act in the body to increase plasma glucose concentrations by increasing hepatic glycogenesis and gluconeogenesis. They also increase protein catabolism and in the periphery, glucocorticoids exert actions that counter the effects of insulin. Glucocorticoids are also

necessary for vascular reactivity. Without them, the vascular smooth muscle becomes unresponsive to epinephrine and norepinephrine, the capillaries expand and their walls become permeable to proteins in the plasma. Finally, glucocorticoids are released in response to stress or noxious stimuli. In animals that lack normal secretion of glucocorticoids, exposure to a stress can be life-threatening (Ganong, 1985).

Secretion of glucocorticoids is regulated by adrenocorticotropin hormone from the pituitary. When a stimulus to ACTH secretion from the hypothalamus reaches the pituitary gland, ACTH is secreted into the general circulation, binds to its receptor at the adrenal gland and stimulates cortisol secretion. Cortisol then acts at target organs to increase plasma glucose levels, etc. However, cortisol also acts at the levels of the brain to reduce ACTH secretion. Cortisol acts at the hypothalamus and pituitary gland negatively to inhibit further ACTH secretion. This is called cortisol negative feedback inhibition of ACTH secretion.

The mechanism of action of cortisol to reduce ACTH secretion has been studied at length in many species and by many investigators. A study by Canny et al. (1989), performed in sheep, examined both hypothalamic and pituitary sites of action for glucocorticoids. Measurements of hypophyseal portal concentrations of AVP and CRF and systemic measurements of ACTH and cortisol concentrations were made before and after dexamethasone infusion with different stimuli to ACTH secretion. The data suggest that glucocorticoids act in a site-specific manner to inhibit ACTH secretion (Canny et al., 1989). During audiovisual stress, hypothalamic CRF and AVP secretion were unaltered but ACTH secretion was inhibited suggesting a pituitary site of action of dexamethasone. In response to hypoglycemia, dexamethasone inhibited both the hypothalamic and pituitary

responses to the stress. Studies in rats have shown that glucocorticoids inhibit CRF secretion as well as CRF synthesis (Sato et al., 1975) suggesting a mechanism for a hypothalamic site of action. On the other hand, glucocorticoids may interfere with CRF activation of second messenger systems (cAMP) at the pituitary and therefore prevent stimulation of ACTH secretion (Bilezikjian and Vale, 1983).

In the fetus cortisol has the ability to inhibit plasma ACTH secretion in response to a hypotensive stimuli by approximately 90 days gestation (Hargrave and Rose, 1985). High concentrations of glucocorticoids near the fetal PVN prevent increased ACTH secretion in response to hypotension and hypoxemia (some effects at the level of CRH- loss of staining in the median eminence, and loss of AVP staining in the external zone of the median eminence; McDonald et al., 1990). Between 117 and 131 days gestation, fetal sheep are extremely sensitive to the negative feedback effects of cortisol. This was demonstrated by infusions of cortisol that caused less than 2ng/ml increases in plasma cortisol concentrations but which completely inhibited the normal ACTH response to hypotension (Wood, 1986). This knowledge predicts the existence of a normal feedback response in the fetus, which is the case in late gestation fetal sheep. However, in near term fetal sheep, cortisol negative feedback regulation of ACTH secretion becomes ineffective. In experiments in which infusions of cortisol increased plasma cortisol concentrations to approximately 60 ng/ml, fetal plasma ACTH secretion was still not suppressed (Wood, 1987, 1988). The mechanism of this reduction in glucocorticoid negative feedback efficacy is not fully understood at present.

2.4 Glucocorticoid Receptor Structure and Function

Biological effects of glucocorticoids are dependent on the presence of functional receptors (Miesfeld et al., 1986). The glucocorticoid receptor is a member of a superfamily of ligand-dependent nuclear transcription factors (Evans, 1988). The glucocorticoid receptor is a transcriptional activator which binds to cis-acting glucocorticoid response element sequences and alters the rate of transcription of specific genes (Yamamoto, 1985; Burnstein and Cidlowski, 1989). Binding studies indicate that the glucocorticoid receptor (as well as other steroid and thyroid receptors) dimerize and bind to their response elements with high affinity (Tsai et al., 1988). Upon activation by hormone binding, the glucocorticoid receptor is thought to interact with one or more components of the transcriptional machinery to increase or decrease the rate of transcription of the genes with which it is associated.

The glucocorticoid receptor has separate and distinct binding domains (Wrange and Gustafsson, 1978; Giguere et al., 1986). DNA binding occurs between amino acids 421-481 on the human receptor and this binding site is highly conserved among steroid receptors (Giguere et al., 1986; Weinberger et al., 1985). This region consists of a clustering of basic amino acid residues which can interact with DNA and a cysteine rich motif which participates in the formation of zinc fingers, another aid in DNA binding (Evans, 1988). Steroid binding occurs approximately between amino acids 500-777 at a single binding site (Giguere et al., 1986; Weinberger et al., 1985). An amino acid region located within the steroid binding domain (approximately a.a. 574-596) of the human glucocorticoid receptor is thought to be involved in direct interaction with a 90 kD heat shock protein (HSP 90) dimer to form the nonactivated 9S complex (Pratt et al., 1988).

Upon binding of glucocorticoid to its receptor and receptor activation, the HSP 90 dimer dissociates from the receptor. However, loss of the HSP 90 dimer before receptor activation results in lower affinity of the glucocorticoid receptor for its ligand (Denis and Gustafsson, 1989). If bases are deleted from the steroid binding domain, the receptor becomes constitutively active (Godowski et al., 1987).

Nonactivated glucocorticoid receptor with a sedimentation coefficient of approximately 9S and a molecular weight of approximately 300 kD under non-denaturing conditions has been found in the cytosol of target cells. Activated complexes have a molecular weight of approximately 100 kD (Sherman and Stevens, 1984). These data support the concept of a HSP 90 dimer binding the nonactivated receptor and dissociating upon activation purely on the molecular weight data alone. It has been postulated that the HSP binding to the receptor maintains proper conformation so after activation by ligand binding, the receptor is able to associate with the DNA. Some studies have suggested that receptor phosphorylation (Orti et al., 1989) and disulfide bond (Silva and Cidlowski, 1989) formation may also be involved in receptor action. Evidence suggests that glucocorticoids are necessary for nuclear translocation (Picard and Yamamoto, 1987) and transcriptional modulation by the glucocorticoid receptor (Picard et al., 1990), but their role in regulating receptor DNA binding is uncertain.

One factor determining the cellular responsiveness to glucocorticoids is the concentration of glucocorticoid receptors (Bougeios and Newby, 1979; Gehring et al., 1986). Glucocorticoids can negatively regulate gene expression, possibly through negative GREs which have been identified in a few genes. Binding of the GR to these GREs results in inhibition of transcription of the gene with which the negative GRE is associated (Beato et al., 1989).

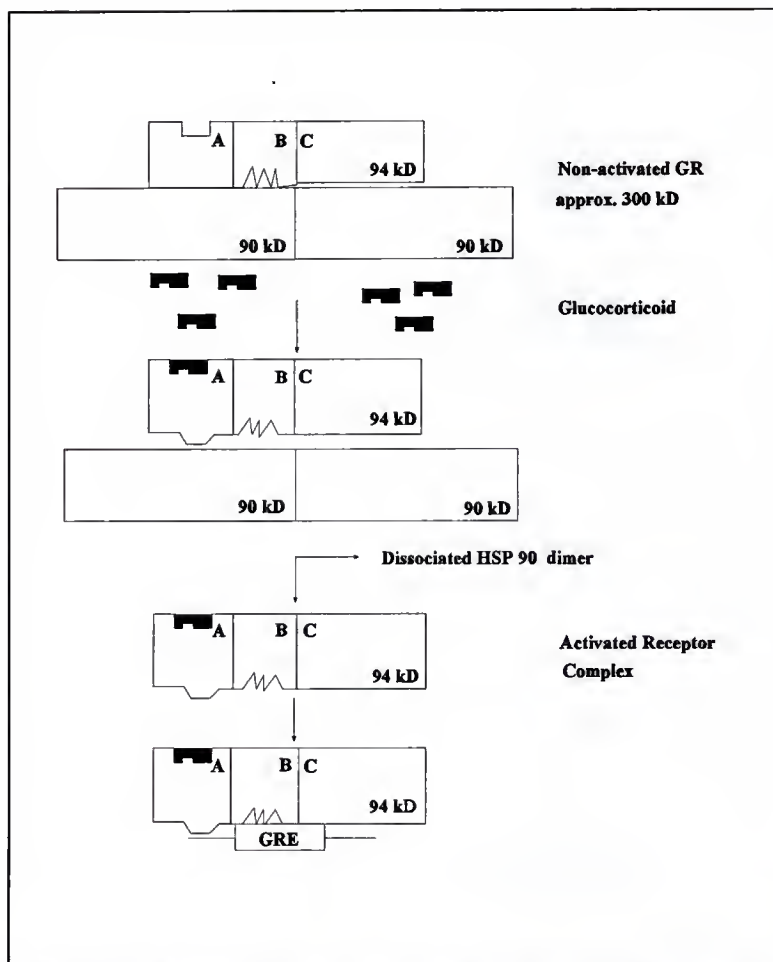


Figure 3: Proposed model for *in vitro* activation of the glucocorticoid receptor (GR) (modified from Denis and Gustafsson, 1989). The non-activated receptor is bound to a dimer of heat shock protein 90 (HSP 90), which masks the DNA binding domain (B). Binding of ligand to the steroid binding domain (A) results in modification of the GR and dissociation of the HSP 90 dimer. The activated receptor complex (as a dimer) is then able to bind DNA at specific sites (GREs - glucocorticoid response elements). Area © on the receptor is the immunogenic region.

2.5 Gonadal Steroid Modulation of ACTH Secretion

It has long been known that female rats have greater activity of the HPA axis than male rats. Studies in adult animals demonstrate that female rats have increased corticosterone secretion in response to stress as compared with male rats. They also had greater corticosterone secretion following ACTH administration and greater adrenal responsiveness to tropic stimulation (Kitay, 1961). As the major difference between the sexes is gonadal steroid production, experiments involving gonadectomy and replacement of gonadal steroids were performed. After gonadectomy, testosterone depressed ACTH content and steroid clearance in male rats but increased adrenal responsiveness to ACTH. In female rats, estradiol had a consistent stimulatory effect on ACTH secretion (Kitay, 1963). In female rats, plasma ACTH and corticosterone responses to restraint stress were enhanced during proestrus, when estradiol concentrations are highest (Kitay, 1963). In ovariectomized (ovx) rats replaced with estradiol, this effect can be restored (Viau and Meaney, 1991). Following ovariectomy, there was a decreased capacity of the pituitary to synthesize ACTH and a decreased responsiveness to stimulation by hypothalamic extracts (Coyne and Kitay, 1969). In ovx rats, estradiol implants into the area of the anterior pituitary, arcuate nucleus and lateral mammillary bodies in rats facilitated pituitary-adrenal activity, suggesting a central nervous system effect (Richard, 1965). However, estrogen stimulation of corticosterone secretion in ovx rats may be due in part to a direct effect on the adrenal cortex (Kitay et al., 1965). In ovx rats, plasma ACTH and corticosterone responses to foot shock and ether vapor stress were lower than in estrogen-replaced ovx rats (Burgess and Handa, 1992). From these results, it was concluded that the increased activity of the pituitary-adrenal axis was due to an impairment of the glucocorticoid negative feedback mechanism.

The mechanism of action of estrogen stimulation of HPA axis activity is not fully understood. Based on the present data, estrogen may interact with a number of systems impinging on the HPA axis. Estrogen uptake has been demonstrated in corticotrophs isolated from anterior pituitary cells from orchidectomized, adrenalectomized rats (Keefer, 1981), suggesting a possible direct effect of estrogen on the pituitary. Estradiol has also been shown to concentrate in tyrosine-hydroxylase containing neurons in the arcuate and periventricular nuclei of the rat (Sar, 1984). In another study, estradiol-concentrating cells have been found in the amygdaloid central nucleus (Beaulieu et al., 1987). Since this dopaminergic system can inhibit ACTH secretion, this may be important in modulating HPA axis activity. In rats given 100 µg estradiol exogenously for two weeks, there was an increase in AVP in SON and PVN of the hypothalamus without any changes in pituitary ACTH and AVP content or basal plasma ACTH or AVP concentrations (Hashimoto et al., 1981). In another study, plasma AVP concentrations were found to be greatest when estrogen concentrations were highest. If the rats were ovariectomized, plasma AVP concentrations decreased but were restored when estrogen was replaced (Skowsky et al., 1979). These data suggest a possible role of hypothalamic releasing factors in the mediation of estrogen stimulation of HPA axis activity.

Androgens have a different effect on the HPA axis. Gonadectomy in male rats increases basal and stimulated plasma ACTH concentrations (Coyné and Kitay, 1971). If androgens are replaced (testosterone or dihydrotestosterone), this increase in plasma ACTH can be reversed (Handa et al., 1994). However, testosterone implants were ineffective in altering corticosterone secretion (Telegdy et al., 1963). Sexual maturation in male rats is accompanied by an increase in POMC gene transcript levels, but testosterone

given to immature male rats had no effect on POMC gene transcript levels (Kerrigan et al., 1992). So it appears that androgens may play an inhibitory role in HPA axis activity. The mechanism of this action is unknown. It has been demonstrated that orchidectomy had no effect on hippocampal, hypothalamic, or pituitary glucocorticoid receptors, suggesting that androgens have no effect on glucocorticoid negative feedback in adult rats (Handa et al., 1994).

2.6 Summary

As has been described, the process of parturition in the fetal sheep is dependent upon the HPA axis of the fetus. Near the end of gestation, a poorly understood signal increases activity of the HPA axis. This has been described as an increase in drive to the HPA axis and is reflected in increased plasma ACTH and cortisol concentrations of the fetus beginning at approximately 120-125 days gestation. Plasma ACTH and cortisol concentrations are further increased in late gestation by a decrease in glucocorticoid negative feedback efficacy at approximately 140 days gestation. This alteration in negative feedback sensitivity appears to be the final step in increasing plasma ACTH secretion and initiating parturition. Understanding this process is crucial in understanding the mechanism of parturition.

In this system, all aspects of the HPA axis seem to have been examined. However, cortisol also increases the production of plasma androgens and estrogens in late gestation. As stated previously, the gonadal steroids have a profound effect on plasma ACTH secretion in the adult animal. We hypothesized that androgens and estrogens may also play a role in pituitary-adrenal axis activity in the fetal sheep. If these gonadal steroids do

have the ability to influence fetal pituitary ACTH secretion, then they may also influence the process of initiation of parturition. The following studies were designed to examine the possibility of a role for gonadal steroids in the control of parturition in the ovine fetus.

CHAPTER 3 GENERAL METHODOLOGY

3.1 Animals and Surgery

The sheep used in these experiments were pregnant ewes (74 days gestation or later) of various breeds (mixed western, Florida native, etc.). Animals were purchased from three suppliers (Institute of Food and Agricultural Sciences, University of Florida, Gainesville, FL; Arborlow Farms, Newberry, FL; Tom Morris, MD). Animals were housed in approved pens in the Health Center Animal Resources Department or at the University of Florida Veterinary School, Metabolic Unit and were maintained under controlled lighting and temperature. Pens were cleaned daily by the husbandry staff and ewes were offered food and water *ad libitum*.

Surgeries were performed using aseptic technique in the Animal Resources Department surgery suites. There were no deaths in these experiments due to the surgical procedure or lack of sterility during the surgery. Ewes were fasted 24 hours prior to surgery and water was withheld for 12 hours. Anesthesia was induced and maintained with 0.5% - 3.0% v/v halothane or isoflurane in oxygen. The abdomen and flank were shorn and aseptically prepared for surgery with povidone iodine. Following intubation ewes were connected to a ventilator. Heart rate, mean arterial pressure, rectal temperature, ventilatory CO₂ and O₂, and respirations were all monitored during the surgery. When ewes were fully recovered, free access to food and water was permitted.

Surgery was performed between days 108 and 125 of gestation, depending on experimental design. The ewes were monitored at all times during the surgery and recovery until the animal could stand under its own effort.

An incision was made along the midline of the abdomen beginning at the umbilicus and extending caudally approximately 10 cm. The hindlimbs of the fetus were located by palpation and the uterine horn containing the hindlimbs was exteriorized. The uterus was incised and a hindlimb was exteriorized from the uterus. An arterial polyvinyl chloride catheter (.030" ID, .050" OD) was placed into the tibial artery and the tip advanced to the descending aorta. A venous polyvinyl chloride catheter (.040" ID, .070" OD) was placed into the saphenous vein and the tip advanced to the inferior vena cava. The limb was returned to the amniotic cavity and the contralateral hindlimb was catheterized as was the first. In each case, the distal end of the vessel was closed with suture ligatures and the catheter was sutured in place with 2.0 silk. A steroid implant or placebo was inserted subcutaneously into the area of the gluteus medius before suturing the second hindlimb. A polyvinyl chloride catheter (.050" ID, .090" OD) with side holes cut into the tip was attached to the second hindlimb with suture for delivery of antibiotics to the amniotic space and for amniotic fluid pressure measurements. The second hindlimb was returned to the amniotic cavity and the uterus was closed with 2.0 silk. 500 mg ampicillin (Polyflex®, Fort Dodge Laboratories, Fort Dodge, IA) was administered into the amniotic cavity and the catheters were exteriorized through a flank incision via trocar. The catheters were filled with heparin (1000 units/ml, Elkins-Sinn, Inc, Cherry Hill, NJ) and closed with a sterile brad inserted into the end. The abdomen was then closed with #3 polyamid suture (Pitman-Moore, France) and 500 mg ampicillin was administered IM to the ewe. A cloth

pouch to house and protect the catheters was fixed to the ewe's flank with #1 polyamid suture. A piece of spandage was then put around the ewes abdomen to prevent the catheters from falling out of the pouch.

Some ewes also received maternal catheters at the time of surgery. An incision was made at the femoral triangle and the femoral artery and vein were exposed and the distal end of the vessel was ligated closed. Polyvinyl chloride catheters (.050" ID, .090" OD) were inserted into both vessels. The arterial catheter was advanced to the descending aorta and the venous catheter was advanced to the inferior vena cava. The catheters were filled with heparin and closed with a sterile nail and exited subcutaneously via a separate flank incision next to the incision for the fetal catheters.

In ewes with twin pregnancies, both fetuses were catheterized if they appeared strong and healthy. If one of the fetuses displayed apparent growth retardation (i.e. was a "runt"), it was not used in the experiments. This was done, in part, because the blood vessels were too small to catheterize. In the case of twins, one twin received the steroid implant and the other twin was a vehicle control. Following surgery, the ewes were treated with 500 mg ampicillin IM BID for 5 days and their temperatures were monitored as an indication of infection.

Steroid pellets were obtained from Innovative Research of America (Toledo, OH). Pellets were 5 mg, 21 day releasing pellets or 15 mg, 60 day releasing pellets (approximately 10 µg per hour). This dose was chosen in order to raise plasma estrogen and androstenedione concentrations to levels that were seen prior to parturition.

The ewes were treated post-operatively with 500 mg ampicillin IM BID for 5 days. Temperatures were also monitored BID for fever as an indication of infection. Post-

operative pain was determined on an individual basis and intervention was needed only in a few cases (veterinary care provided by Animal resources).

3.2 Blood Collection and Handling

Catheters were removed from the cloth pouch and the distal ends were scrubbed with povidone iodine (Betadine®, Purdue Frederick Co., Norwalk, CT) and alcohol. The brad was removed and a sterile blunt adapter was inserted. Blood was withdrawn until it replaced the catheter dead space (about 3 ml in the fetus and about 5 ml in the ewe). The resultant fluid was discarded. Blood (3-5 ml) was then drawn and collected into chilled tubes containing Na_4EDTA (57 μg or .3M EDTA/ml blood)(Sigma Chemical Co., St. Louis, MO). Blood gas samples (1.5 ml) were drawn anaerobically into syringes coated with heparin for measurement of fetal blood gases and pH (BMS3MK2 blood microsystem and PHM73 pH and blood gas analyzer, 37°C, Radiometer, Copenhagen, Denmark). After sampling, the blood volume removed was replaced with 0.9% normal saline (Baxter Healthcare Co., Deerfield, IL) and the catheters refilled with heparin. Catheters were then plugged with sterile brads and returned to the cloth pouch. Each day the catheters were opened, the fetus was administered 750 mg ampicillin via the amniotic catheter.

Blood samples were kept on ice until transported to the lab (approximately 1-4 hours). Samples were centrifuged at 3000 x g for 20 minutes at 4°C in a refrigerated centrifuge (Sorvall RT6000B, Dupont, Newtown, CT). After centrifugation, the plasma was transferred to a clean tube and stored at -20°C until hormones were assayed.

3.3 Infusion Experimental Procedures

Experiments were performed between days 126 and 135 days gestation. All experiments were started between 0900 and 1100 hours to minimize possible variation in hormone concentrations between experiments. On the morning of the experiment, ewes were loaded into a study cart and transported to a study room (either in the animal resources facility or in the laboratory). Ewes remained in the cart for the duration of sampling. Catheters were then flushed with heparinized saline (2.0% v/v) and connected to transducers (Statham P231d, Statham Instruments, Oxnard, CA) for measurement of fetal arterial and amniotic pressure. At least one hour was then allowed for the ewe to adjust to the new environment. Arterial and amniotic fluid pressures were measured during the final 20 minutes of the experiment using a Grass Model 7 recorder. The data were digitized and stored using an IBM AT microcomputer and a Keithley analog-to-digital converter on-line.

The experiments used to examine fetal ACTH responses were designed as "feedback experiment." The fetal plasma ACTH response to a stress (in this case, hypotension) was elicited by infusion of nitroprusside. This response is reproducible and controllable by infusing at a known dose and rate. The dose and rate selected decreased fetal mean arterial blood pressure significantly (approximately 50%). In order to assess feedback efficacy of the hypothalamus-pituitary-adrenal axis, fetal plasma ACTH response was examined under control conditions with an infusion of normal saline prior to the hypotension stress. This response was then compared to the fetal plasma ACTH response following infusion of cortisol (hydrocortisone sodium succinate, Upjohn, Kalamazoo, MI) (0.20 ng/ml in 0.9% saline) infused into to fetal inferior vena cava at a rate of 5.1 ml/hr for

2 hr. This dose of cortisol inhibited the fetal HPA axis and decreased fetal plasma ACTH secretion by approximately 50% in control fetuses. By comparing the control plasma ACTH response to the inhibited response following the cortisol infusion, an estimate of feedback efficacy was obtained.

The stimulus for fetal plasma ACTH secretion was sodium nitroprusside (NiPr, Elkins-Sinn, Inc., Cherry Hill, NJ). NiPr is a nitric oxide (NO) donor. The NO acts on the smooth muscle vascular epithelium causing relaxation and a subsequent decrease in MAP. This decrease in MAP is sensed by the carotid sinus baroreceptors as a decrease in stretch. This decrease in MAP results in decreased firing of impulses from the carotid sinus through the nerve of Hering to the glossopharyngeal nerve to the nucleus of the tractus solitarius in the medulla (Berne, Levy, 1986). In addition to increasing sympathetic tone to restore blood pressure, the NTS also has projections to the hypothalamus. With a decrease in MAP, the NTS signals the hypothalamus and plasma ACTH secretion (as well as AVP) is increased. NO acts through its second messenger, guanylate cyclase, to increase cGMP, which may also be an independent stimulus for ACTH secretion.

In each experiment, vehicle (0.9% w/v normal saline) or cortisol was infused into the fetal inferior vena cava at a rate of 5.1 ml/hr for 2 hours. One hour after the end of the vehicle or cortisol infusion, NiPr was infused into the fetal inferior vena for 10 minutes at a rate of 50 μ g/min (0.5 ml/min) using a constant infusion syringe pump (Sage Instruments, Orion Research, Inc., Model 341A, Cambridge, MA). Blood sample (5 ml) was drawn from the fetal arterial catheter at the beginning of the vehicle or cortisol infusion. Blood samples (3 ml) were drawn from the fetus after 1 and 2 hours of vehicle

or cortisol infusion and 0, 10, 20 minutes after the start of the NiPr infusion. Blood samples were handled as described above.

3.4 Hormone Assay Techniques

Plasma ACTH concentrations were measured by radioimmunoassay (RIA) as previously described (Bell et al., 1991) using an antibody raised in rabbits developed in this laboratory to human-ACTH(1-24). Iodinated ACTH (I^{125} -ACTH) was prepared using the chloramine-T method (Berson and Yalow, 1968) with human-ACTH (1-39) (Sigma, St. Louis, MO) and radioactive sodium iodide (Amersham, Arlington Heights, IL). I^{125} -ACTH was made fresh approximately every 6 weeks. ACTH was first extracted from plasma before assaying. ACTH (500 μ l) was extracted on glass (35 mg per tube)(100-200 mesh glass, Corning Glass Works, Corning, NY) in 500 μ l assay buffer (.05M phosphate buffer, pH 7.4, with 0.2% w/v silicic acid-extracted bovine serum albumin (BSA, Sigma, #9647). The supernatant was aspirated and the glass washed with assay buffer. The ACTH was eluted from the glass with 1 ml acid:acetone (1 volume 0.25N HCL: 1 volume acetone). The extracts were dried under vacuum (Savant Instruments, Farmingdale, NY) and frozen at -20°C until assayed. Extracts were reconstituted overnight in 500 μ l assay buffer (containing 0.5% v/v mercaptoethanol). Extraction recovery was corrected by comparing samples to a standard curve prepared from standard extracted with each set of samples.

Plasma AVP concentrations were measured as previously described (Raff et al, 1991) using an AVP antibody raised in rabbits. Iodinated AVP was purchased from Amersham and synthetic AVP from Sigma. AVP was extracted from 500 μ l plasma with

1 ml bentonite slurry (0.3% w/v in distilled water) and acidified with 500 μ l 1N HCl. Extracts were eluted with 1 ml acid:acetone (20% 1N HCl: 80% acetone) with sonication. Samples were then evaporated to dryness and stored at -20°C until assayed. Extracts were reconstituted with 250 μ l assay buffer (0.05 M phosphate buffer, pH 7.4 with 0.01 M EDTA (Sigma, #ED4SS) and 0.2% BSA w/v (Sigma, #A-7638)). Extraction recovery was corrected by comparing samples to a standard curve prepared from standard extracted with each set of samples.

Plasma cortisol concentrations were measured as previously described (Wood et al., 1993) using an antibody raised in rabbits and tritiated cortisol purchased from Amersham (#TRK-407) and cortisol standard from Sigma. Cortisol was extracted from 20 μ l plasma (in duplicate) with 1 ml ethanol. Standard was prepared in ethanol, and standards and samples were dried under vacuum with heat and immediately reconstituted with 500 μ l assay buffer [0.05 M phosphate buffer (using 0.06 M sodium phosphate dibasic and 0.04 M sodium phosphate monobasic) pH 7.0, with 0.15 M NaCl, 0.1% w/v gelatin, and 0.1% w/v sodium azide].

Plasma estrogen concentrations were measured as previously described (Kalra and Kalra, 1974). 500 μ l plasma was extracted twice in duplicate with 2 ml ethyl ether. Extracts were dried under air and reconstituted to 200 μ l with assay buffer (0.1M phosphate buffer, pH 7.0, with 0.1% w/v gelatin, 0.9% w/v sodium chloride, and 0.1% w/v sodium azide). Rabbit antiserum #244 was used at a dilution of 1:50,000 for a final assay dilution of 1:200,000. Tritiated estradiol was obtained from Dupont NEN (# NET-317). This antibody cross-reacted with estradiol (approximately 10%) and estrone, therefore results were expressed as plasma estrogen concentrations. This antibody also

cross-reacted with a substance in sheep plasma which resulted in a background reading of 6-10 pg/ml in plasma from ovariectomized (ovx) ewes. When the plasma was stripped with charcoal, this interference was eliminated. To correct for this blank problem, a pool of ovx plasma was evaluated in each assay. The value obtained from this pool was then subtracted from values obtained for the unknowns and results were calculated. A pool of maternal plasma was also included as an internal standard.

Due to the normal physiological variations in plasma estrogen values and the high background interference in the assay, plasma estradiol concentrations were further analyzed after separation of estradiol from estrone. 1 ml plasma from each fetus (estradiol-treated and sham-control) was extracted as previously described. After evaporation, each sample was reconstituted in the solvent system used in the Quik-Sep® LH-20 steroid separation system (Isolab inc., Akron, OH). Each sample was fractionated by passing through the columns, the appropriate fractions collected, evaporated to dryness, and reconstituted with assay buffer. Samples were then run in the estrogen assay as before. This measurement was used to estimate the increase in plasma estradiol levels by the administration of the steroid pellets. The plasma estrogen levels were used in the ontogeny analysis, since the plasma estradiol was measured for only a single time point in each fetus.

Tissue CRF concentrations were measured as previously described (Keller-Wood and Wood, 1991) using an antibody to ovine CRF (oCRF). Iodinated oCRF was purchased from Amersham and oCRF standard from Bachem (Torrance, CA). Tissue samples were diluted in assay buffer (0.05 M phosphate buffer, pH 7.4 with 0.01 M EDTA, 0.2% BSA w/v (fraction V, #A-7638), 0.1% w/v sodium azide, and 0.05% v/v

Triton X-100) and run unextracted. Samples from G-75 column were evaporated to dryness and reconstituted in assay buffer to be run in the assay.

Plasma androstenedione concentrations were measured using a kit from Diagnostic Products Corporation, Los Angeles, CA. 500 μ l plasma was extracted with 5 ml ethyl acetate and hexane (3:2 ratio, respectively). The liquid phase was frozen and the organic phase was transferred to a clean tube and evaporated to dryness in a warm water bath under nitrogen. Samples were then reconstituted in 600 μ l assay buffer (provided in kit) and 200 μ l aliquots were assayed.

3.5 Tissue Collection and Handling

After experimentation, the ewes, fetuses and lambs were euthanized with sodium pentobarbital (minimum of 1 grain/kg). Tissues were dissected as quickly as possible, placed in a clean tube and quick frozen in a dry ice and acetone bath. Samples were stored at -20°C until processed. Tissues were later homogenized (Tekmar, Cincinnati, OH) in Laemmli buffer (Laemmli, 1970) (0.0625 M Tris with 2% w/v SDS, 10% v/v glycerol, 0.72 M mercaptoethanol, and 0.3% w/v PMSF) in a ratio of 5 ml buffer per 1 g tissue. Homogenates were centrifuged at 15,000 $\times g$ for 20 minutes at 4°C to remove particulate matter and supernatant was stored at -40°C until analyzed by RIA or Western blotting. Protein concentration of each sample was determined by the method of Bradford (Bradford, 1976) using a kit purchased from BioRad (# 500-0002, Hercules, CA). Results were expressed or corrected for the protein concentration in each sample.

Tissue samples were run on pre-cast polyacrylamide gels (BioRad) with 20-40 μ g protein loaded per lane for each sample. Molecular weights were estimated by comparing

bands to known molecular weight proteins (Amersham, Rainbow coloured markers, #RPN-755 and #RPN-756) which were run on each gel. Separation of the proteins were performed using a Mini-Protean® II cell gel electrophoresis system (BioRad) at approximately 100 V. Proteins were transferred to membranes overnight at 22 V. Membranes were blocked with a 7.5% w/v non-fat powdered milk solution and stained using anti-rabbit Ig, horseradish peroxidase, linked whole antibody (raised in donkey, #Na-934, Amersham) diluted 1:700 and Renaissance™ western blot chemiluminescence reagent (Dupont). Individual protocols based on the protein being identified are as follows:

Glucocorticoid receptor - Samples were run on 10% (#161-0907) stacking gels.

Primary antibody was obtained from Affinity BioReagents (PA1-511, Neshanic Station, NJ) and diluted to 10µg per blot for incubation.

Corticotropin-releasing factor - Samples were run on 15% (161-0908) stacking gels. Primary antibody was the same as was used in the RIA. A dilution of 1:80,000 was used for incubation.

Arginine vasopressin - Samples were run on 15% stacking gels. Primary antibody was the same as was used in the RIA. A dilution of 1:34,000 was used for incubation.

Adrenocorticotrophic hormone - Samples were run on 16.5% (#161-0922) tris-tricine gels. Primary antibody was obtained from Zymed Laboratories (# 18-0087, S. San Francisco, CA) and diluted 1:250 for incubation.

CHAPTER 4

MODULATION OF OVINE FETAL ADRENOCORTICOTROPIN SECRETION BY ANDROSTENEDIONE AND 17 β -ESTRADIOL

4.1 Introduction

In fetal sheep, parturition is stimulated by increased fetal hypothalamus-pituitary-adrenal (HPA) axis activity (Challis and Brooks, 1989; Liggins et al., 1973). Parturition can be prevented or delayed by destruction of the pituitary (Liggins et al., 1966, 1967; Liggins and Kennedy, 1968) or stimulated by infusions of ACTH (Liggins, 1968, 1969) or glucocorticoids (Jack et al., 1975; Wood and Keller-Wood, 1991). Between 100 and 125 days of gestation, fetal plasma ACTH and cortisol concentrations are low; during this time a majority of the cortisol in fetal plasma originates from the maternal adrenal. Fetal adrenal cortisol secretion slowly increases between 125 to 135 days of gestation. After 135 days of gestation, ACTH and cortisol continue to increase in a semilogarithmic pattern (Bassett and Thorburn, 1969; Rase et al., 1978; Wintour et al., 1975; Wood, 1988) until parturition occurs at approximately 145 days (term in the chronically instrumented animal). Increasing concentrations of cortisol in fetal plasma act at the placenta to induce the enzyme, cytochrome P450_{C17}, which has 17 α -hydroxylase and 17, 20 lyase activities. Induction of this enzyme increases the production of androgens and estrogens from progesterone (Anderson et al., 1975; Pomerantz and Nalbandov, 1975; Steele et al., 1976; Yu et al., 1983). The resultant increase in estrogen-to-progesterone

ratio increases uterine contractility and eventually culminates in initiation of labor and parturition (Liggins, 1974).

Understanding the mechanism of the increased fetal HPA axis activity at the end of gestation is key to understanding the mechanism of spontaneous parturition in this species. It has been previously demonstrated that fetal sheep are very sensitive to cortisol negative feedback of ACTH secretion between 120 and 130 days (Wood, 1986) but then become relatively insensitive to the effects of cortisol near term (Wood, 1988). It has been proposed that this decrease in cortisol negative feedback efficacy after 135 days of gestation may be important for the generation of the preparturient increase in plasma ACTH and cortisol (Wood, 1988). The mechanism of this change in negative feedback has not been identified. It is possible that increased fetal plasma estrogen and/or androgen concentrations might be involved.

Studies in adult animals have shown that estrogen increases the activity of the HPA axis. Female rats have higher basal and stimulated plasma corticosterone levels than males rats (Kitay, 1961) and female rats have enhanced plasma ACTH and corticosterone responses to stress during proestrus (Viau and Meaney, 1991). Several groups have shown in rats that ovariectomy results in decreased plasma ACTH bioactivity and corticosterone responses to stress that can be reversed by exogenous estrogen replacement (Burgess and Handa, 1992; Coyne and Kitay, 1969; Kitay, 1963). Because estrogens and androgens increase prior to parturition and estrogens are known to interact with the HPA axis, we propose that androgens or estrogens might influence fetal ACTH secretion at the end of gestation.

These experiments were designed to investigate the effects of exogenous androstenedione and 17β -estradiol on ACTH secretion in late gestation fetal sheep.

These steroids were chosen because they are naturally-occurring steroids with known increased concentrations at the end of gestation. Specifically, we tested the hypothesis that elevated fetal androstenedione or estradiol would result in increased plasma ACTH secretion through altered cortisol negative feedback.

4.2 Materials and Methods

Animals. Twenty five pregnant ewes of known gestational ages were studied. Seventeen of these ewes were included in all aspects of this protocol. An additional eight ewes were included as supplementary animals used to estimate the effect of hormone pellet implantation on fetal plasma steroid concentrations. Of the animals subjected to the complete protocol, 11 ewes carried singleton fetuses and 6 ewes carried twins. In the supplementary group, all 8 ewes carried singleton fetuses.

Fetal surgery. Aseptic surgery was performed for chronic implantation of vascular and amniotic fluid catheters and steroid pellets on day 122 ± 0.9 (estradiol-treated group), 120.0 ± 1.1 (androstenedione treated group) or day 122.4 ± 1.0 (control group) days of gestation (term is approximately 148 days in uncatheterized fetal sheep). Surgery was performed as previously described (Wood, 1986, 1988). At the time of catheter placement, a pellet containing 5 mg androstenedione or 17β -estradiol (Innovative Research of America, Toledo, OH) was implanted subcutaneously in the area of the gluteus medius. Sham control fetuses were catheterized but did not receive a pellet implant. Each fetus received 750 mg ampicillin via the amniotic fluid at the time of surgery and again each day the vascular catheters were flushed. The ewes received 750 mg ampicillin IM post-operatively BID for 5 days. Catheters were flushed and

reheparinized at the time of blood collection or experimentation (at least once every 3 days).

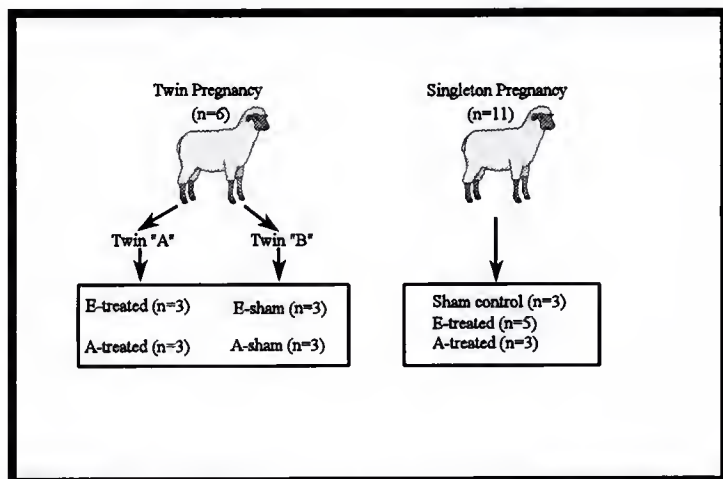


Figure 4.1. Distribution of experimental animals.

The distribution of singleton and twin pregnancies and the distribution of estradiol, androstenedione, or sham treatments are illustrated in Figure 4.1. In the 6 ewes carrying twins, one twin received a pellet and the other twin was a sham control (3 estradiol and 3 androstenedione, each with a twin control). Eight singleton fetuses received pellets (5 estradiol and 3 androstenedione) and three were sham controls.

Experimental protocol. Twenty-three fetuses were subjected to an experimental protocol designed to test fetal ACTH responsiveness to stimulation and to test for alterations in cortisol negative feedback sensitivity (previously described in Chapter 2). The experimental design is illustrated in Figure 4.2. Briefly, vehicle (0.9% w/v saline) or cortisol (HSS: hydrocortisone sodium succinate; 6.0 µg/ml in 0.9% w/v saline)

was infused into the fetal inferior vena cava at a rate of 5.1 ml/hr for 2 h. One hour after the end of the vehicle or HSS infusion, NiPr was infused into the fetal inferior vena cava for 10 min at a rate of 50 μ g/min (0.5 ml/min) using a constant infusion syringe pump. Blood samples (5 ml) were drawn from the fetal arterial catheters at the beginning of the vehicle or HSS infusion. Blood samples (3 ml) were drawn from the fetus after 1 and 2 h of vehicle or HSS infusion and 0, 10, and 20 min after the start of the NiPr infusion. Blood samples were placed into chilled tubes containing EDTA. An additional fetal arterial blood sample (1 ml) was drawn anaerobically at the beginning of each experiment for measurement of fetal blood gases and pH. Arterial and amniotic fluid pressures were measured during the final 20 min of the experiment. The data were digitized and stored

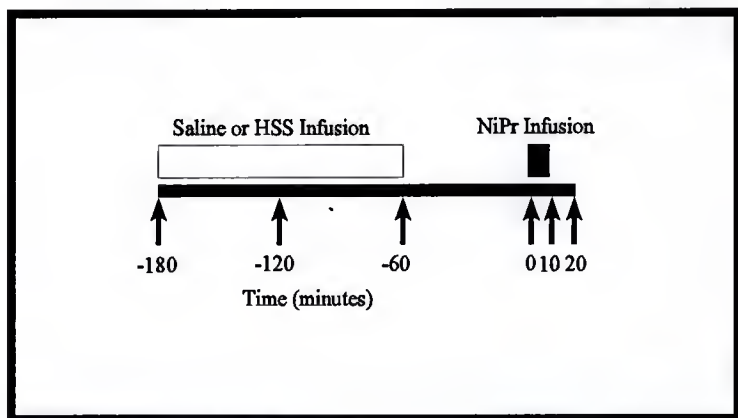


Figure 4.2. Experimental design.

using an IBM AT microcomputer and a Keithley analog-to-digital converter on-line.

Not all of the 23 fetuses included in this protocol were subjected to both saline and HSS infusion experiments. Seven fetuses were subjected to only one experiment because of spontaneous delivery ($n=3$), complications not related to the experiment ($n=3$), and

pump failure (n=1). Although not all fetuses were studied in all parts of the experimental design, all fetuses were healthy as defined by pH, PO₂, and PCO₂ when they were studied (Table 4.1).

A supplemental group of 8 fetuses were subjected to surgical implantation (n=2) or sham-implantation (n=6) of androstenedione pellets and were allowed to recover from surgery, as described above. These fetuses were not subjected to saline, HSS, or nitroprusside infusion. They were used only to test the effect of pellet implantation or sham pellet implantation on fetal plasma steroid concentrations. The addition of these fetuses was necessary because we needed larger group sizes in order to increase the statistical power in the assessment of statistical significance when analyzing fetal plasma concentrations of estradiol and androstenedione.

Analysis of blood samples. Plasma ACTH, cortisol, and estradiol concentrations were measured by RIA as previously described (Bell, et al., 1991; Kalra and Kalra, 1974; Wood, et al., 1993). Plasma androstenedione concentrations were measured using a commercial RIA kit (Diagnostic Products Corporation, Los Angeles, CA). Plasma ACTH and cortisol concentrations were measured in all plasma samples; androstenedione and estradiol were measured in the first plasma sample drawn in each experiment. In the fetuses subjected to the complete protocol, the volume of plasma available for analysis was limited, therefore we measured plasma androstenedione concentrations in all fetuses treated with androstenedione, but only in 3 sham fetuses. To augment the number of fetuses available for measurement of plasma steroid concentrations after pellet implantation, we added a supplemental group of 2 androstenedione-treated fetuses and 6 sham fetuses. All estradiol samples were run in a single assay, as were the androstenedione samples.

Calculations and statistical analyses. Fetal mean arterial pressure was corrected by subtraction of amniotic fluid pressure. Data were analyzed using one- and two-way analysis of variance (ANOVA) for equal or nearly equal "n" corrected for repeated measures in one dimension (time) (Winer, 1971). The two-way ANOVA was used to compare values of experimental variables in saline- and HSS-infusion experiments within each treatment group (i.e., control, androstenedione, and estradiol treatment groups). *A posteriori* pairwise testing of differences between group means was performed using Duncan's multiple range test (Duncan, 1957). A significance level of 0.05 was used to reject the null hypothesis in all tests. Values are reported as the mean $\pm$ SEM.

4.3 Results

Blood gases. Fetal arterial blood gases and pH measured prior to the start of experiments are reported in Table 4.1. The values in each group are similar to blood gases that we have reported in healthy fetuses in previous experiments (Wood, 1986; Wood, 1988). There was no statistically significant differences in any of the measurements between any of the treatment groups (data are mean $\pm$ SEM).

Table 4.1. Fetal blood gases and pH at the time of experimentation.

	P _a O ₂ (mmHg)	P _a CO ₂ (mmHg)	pH _a
Androstenedione + saline (n=6)	21.3 $\pm$ 1.5	49.8 $\pm$ 3.1	7.348 $\pm$ 0.008
Androstenedione + HSS (n=6)	22.1 $\pm$ 1.5	48.8 $\pm$ 2.9	7.334 $\pm$ 0.009
Estradiol+saline (n=6)	24.2 $\pm$ 2.1	43.2 $\pm$ 1.0	7.331 $\pm$ 0.023
Estradiol + HSS (n=7)	21.3 $\pm$ 1.6	43.1 $\pm$ 0.9	7.346 $\pm$ 0.014
Sham + saline (n=8)	25.1 $\pm$ 1.2	45.7 $\pm$ 2.2	7.337 $\pm$ 0.008
Sham + HSS (n=8)	21.4 $\pm$ 1.2	45.0 $\pm$ 2.6	7.350 $\pm$ 0.012

Fetal plasma estradiol and androstenedione concentrations. Plasma estradiol concentrations were significantly increased in estradiol-treated fetuses (39.1 ± 11.1 pg/ml, $n=7$) compared with sham-treated fetuses (11.4 ± 3.9 pg/ml, $n=12$). Similarly, fetal plasma androstenedione concentrations in androstenedione-treated fetuses (273.1 ± 20 pg/ml, $n=9$) were significantly elevated when compared to sham-treated fetuses (203.5 ± 10 pg/ml, $n=8$). The breakdown of twin and single fetuses along with their corresponding estradiol or androstenedione concentrations are shown in Table 4.2. There was no significant difference in steroid plasma concentrations between the twin control fetuses and the single sham-control fetuses, indicating no significant steroid transfer in utero between the treated twin and the control twin.

Table 4.2. Plasma steroid concentrations in the twin and singleton fetuses.

	Plasma Estradiol (pg/ml)	Plasma A.Dione (pg/ml)
Steroid-Treated Twin	58.5 ± 26.1 ($n=3$)	238.6 ± 25.3 ($n=4$)
Control Twin	21.1 ± 10.0 ($n=3$)	209.4 ± 21.8 ($n=4$)
Steroid Singleton	27.4 ± 6.9 ($n=5$)	307.6 ± 27.3 ($n=4$)
Control Singleton	8.1 ± 3.7 ($n=9$)	198.8 ± 8.1 ($n=5$)

Basal fetal plasma ACTH and cortisol concentrations. Basal or initial plasma concentrations of ACTH and cortisol (prior to the start of saline infusion) are illustrated in Figure 4.3. Estradiol treatment significantly increased fetal plasma ACTH (top panel) and cortisol (bottom panel) concentrations ($p < 0.05$). Androstenedione treatment did not significantly alter fetal plasma concentrations of either ACTH or cortisol (data are represented as mean $\pm$ SEM).

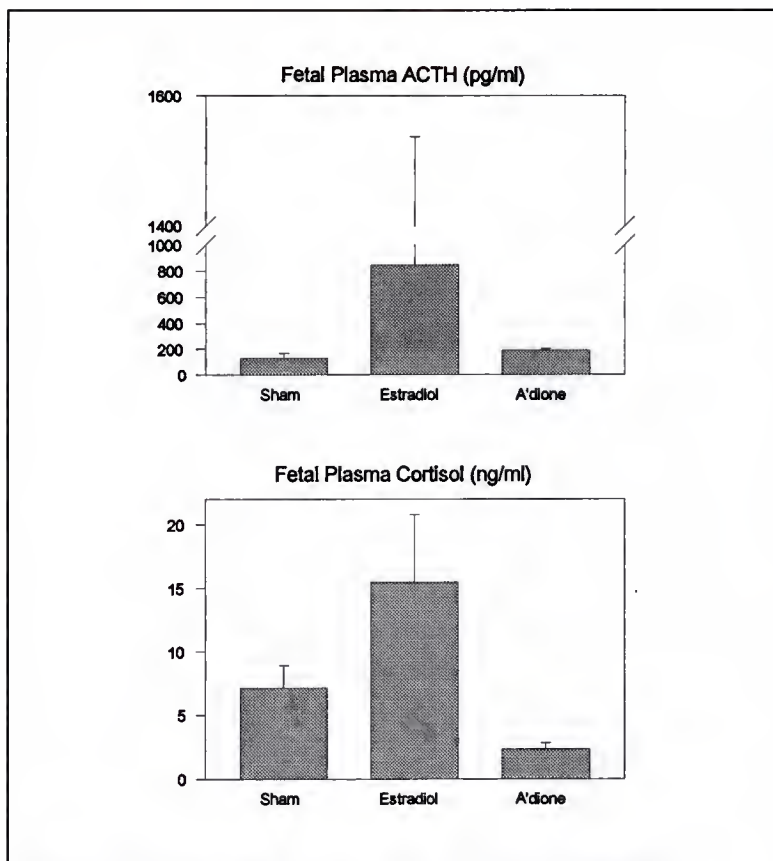


Figure 4.3. Basal fetal plasma ACTH (top panel) and cortisol (bottom panel) concentrations in sham-, estradiol-, and androstenedione-treated fetuses at time -180 in the saline-infused animals. Data are represented as mean values and error bars represent 1 SEM.

ACTH and cortisol responses to saline or HSS infusion. Fetal plasma cortisol concentrations before and during a two-hour infusion of HSS or saline are reported in Table 4.3. In both the sham control and the androstenedione-treated fetuses, HSS

infusion produced statistically significant increases and saline infusion did not produce any changes in fetal plasma cortisol concentration. In the estradiol-treated fetuses, however, there were spontaneous, statistically significant ($p < 0.05$), increases in fetal plasma concentrations of cortisol during the infusion of saline. Because of this apparently spontaneous activity of the HPA axis, we measured no apparent increase in fetal plasma cortisol concentration during cortisol infusion, but a paradoxical increase during saline infusion (Table 4.3a). The apparent increase in fetal plasma cortisol during saline infusion was accounted for by spontaneously increased fetal HPA axis activity in 3 of the 5 estrogen-treated fetuses in this experimental group.

Table 4.3. Plasma cortisol (table 4.3a) and ACTH (table 4.3b) concentrations during HSS or saline infusions. Data are expressed as mean $\pm$ SEM.

Table 4.3a. Fetal plasma cortisol concentrations (ng/ml).

TIME (MIN)	A'Dione + Saline	A'Dione +HSS	Estradiol +Saline	Estradiol +HSS	Control +Saline	Control +HSS
-180	2.32 $\pm$ 0.5	3.61 $\pm$ 1.0	15.5 $\pm$ 4.9	17.4 $\pm$ 2.5	6.10 $\pm$ 1.2	8.00 $\pm$ 2.0
-120	1.91 $\pm$ 0.4	7.07 $\pm$ 3.6	25.4 $\pm$ 9.8	17.9 $\pm$ 3.0	7.04 $\pm$ 1.9	14.1 $\pm$ 3.6
-60	1.74 $\pm$ 0.2	7.35 $\pm$ 3.2	29.3 $\pm$ 13.4	15.6 $\pm$ 2.3	5.62 $\pm$ 1.6	13.1 $\pm$ 3.4
0	2.94 $\pm$ 0.8	3.37 $\pm$ 1.1	27.2 $\pm$ 13.6	16.7 $\pm$ 2.3	7.12 $\pm$ 1.7	9.71 $\pm$ 2.1

Table 4.3b. Fetal plasma ACTH concentrations (pg/ml).

TIME (MIN)	A'Dione + Saline	A'Dione +HSS	Estradiol +Saline	Estradiol +HSS	Control +Saline	Control +HSS
-180	199 $\pm$ 13	182 $\pm$ 15	846 $\pm$ 618	123 $\pm$ 27	127 $\pm$ 39	88 $\pm$ 21
-120	192 $\pm$ 17	167 $\pm$ 14	1508 $\pm$ 786	94 $\pm$ 19	107 $\pm$ 28	142 $\pm$ 22
-60	156 $\pm$ 15	187 $\pm$ 11	1444 $\pm$ 569	117 $\pm$ 37	109 $\pm$ 33	163 $\pm$ 17
0	195 $\pm$ 34	182 $\pm$ 13	707 $\pm$ 423	129 $\pm$ 17	172 $\pm$ 41	111 $\pm$ 20

Fetal plasma ACTH was not significantly altered in any of the groups between -180 and 0 min, except in the estradiol-treated fetuses (Table 4.3b). During saline infusion, the estradiol-treated fetuses exhibited an apparent increase (but not statistically significant) in plasma ACTH concentrations compared to the HSS infused fetuses at the -120 and -60 min time points. The increases in both ACTH and cortisol concentrations in the plasma of these fetuses was accounted for by apparently spontaneous increases in three of the five fetuses.

Stimulus to fetal ACTH secretion: blood pressure responses to nitroprusside.

Nitroprusside infusion produced hypotension to a similar degree in both infusion groups (saline vs. HSS) in each treatment group (Figure 4.4: sham, estradiol, and androstenedione groups are shown in top, middle, and bottom panels, respectively). HSS infusion tended to increase fetal mean arterial blood pressure in all treatment groups, although this effect was statistically significant only in the androstenedione-treated fetuses.

ACTH and cortisol responses to nitroprusside infusion. The plasma ACTH and cortisol responses to the nitroprusside infusion are shown in Figures 4.5 and 4.6.

Nitroprusside infusion significantly increased plasma ACTH concentrations following saline infusion (peak values of 1237 ± 311 pg/ml, 2239 ± 874 pg/ml, and 1045 ± 429 pg/ml in androstenedione-, estradiol- and sham-fetuses at 10 min, respectively). Comparison of the peak fetal plasma ACTH values in the sham- vs. estradiol-treated fetuses revealed that estradiol treatment significantly augmented the ACTH response to nitroprusside.

Comparison of peak values in sham- and androstenedione-treated fetuses did not reveal any significant alteration of the responsiveness to nitroprusside by androstenedione.

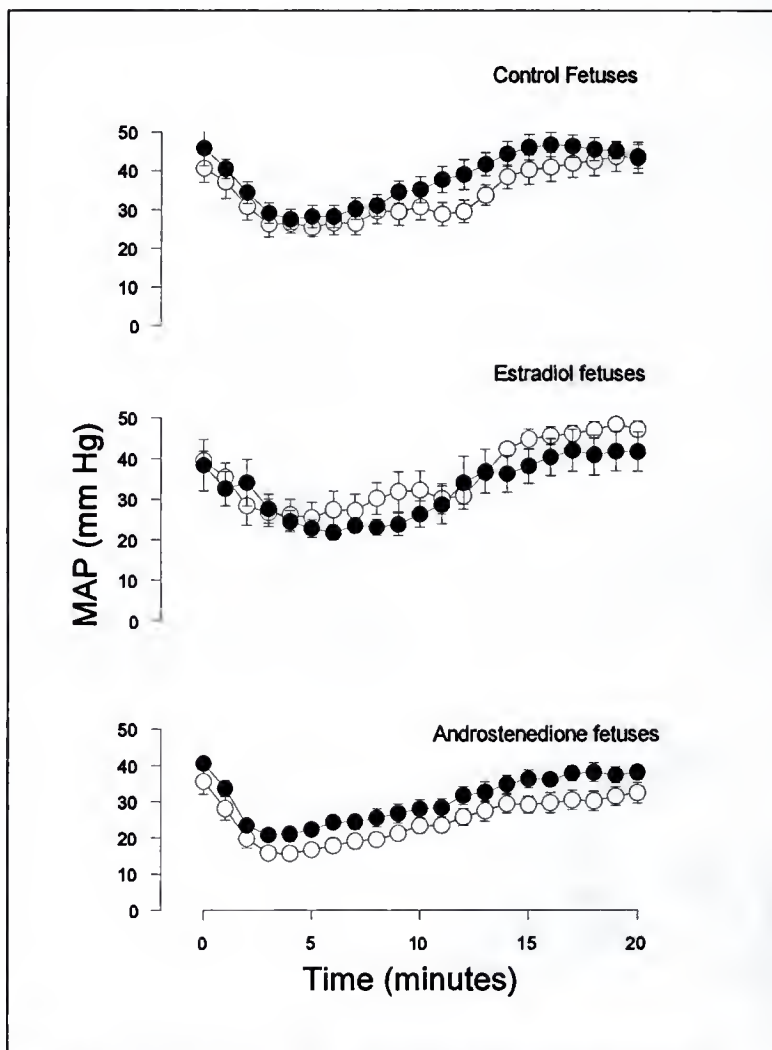


Figure 4.4. Fetal mean arterial pressure responses to nitroprusside infusion in sham-control, estradiol, and androstenedione fetuses following 2 hour saline infusion (O) or HSS (●) infusion. Nitroprusside was infused between 0 and 10 min. Data are represented as mean $\pm$ SEM.

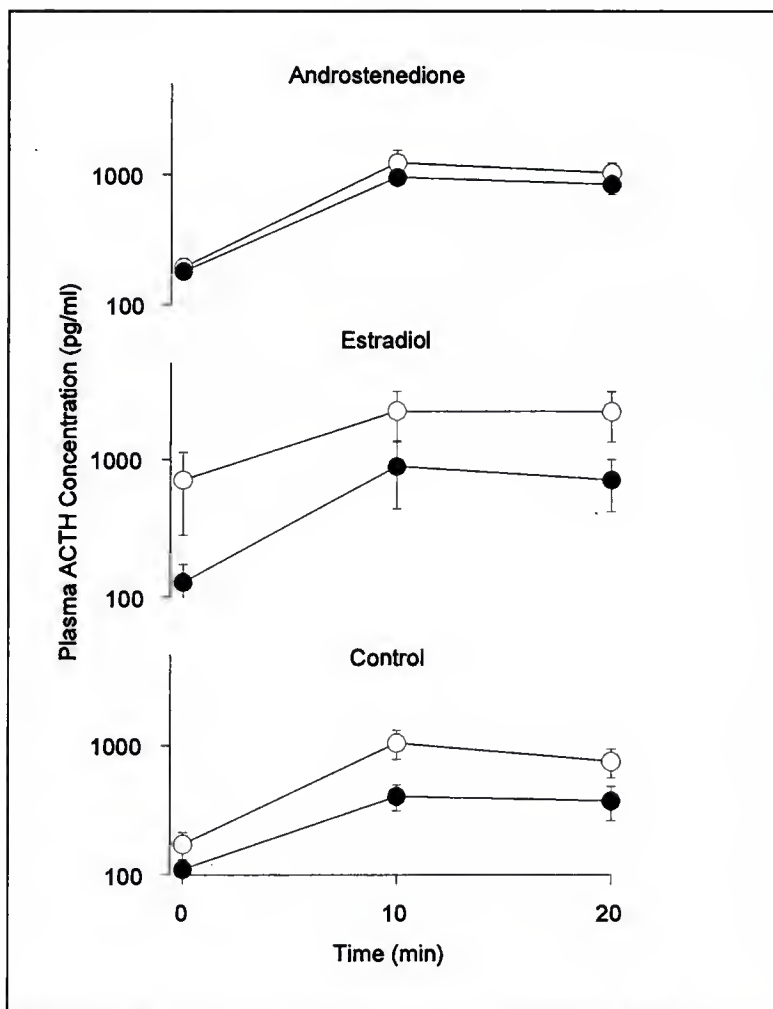


Figure 4.5. Fetal plasma ACTH responses (log scale) to nitroprusside infusion in androstenedione, estradiol, and sham control fetuses following 2 hour saline (○) or HSS (●) infusion. Nitroprusside was infused between 0 and 10 minutes. Data are represented as mean $\pm$ SEM.

The ACTH response to nitroprusside was significantly reduced by HSS infusion in the sham control group when analyzed by two-way ANOVA corrected for repeated measures. However, this analysis was not able to demonstrate statistical significance of the effect of HSS on fetal ACTH responses to nitroprusside in either the estradiol-treated fetuses or in the androstenedione-treated fetuses. When the sham control and estradiol-treated fetuses were analyzed together in a three-way ANOVA corrected for repeated measures, there was a significant interaction effect of HSS infusion and experimental treatment (sham control vs. estradiol treatment). A similar comparison of the androstenedione- and sham-treated fetuses by three-way ANOVA also demonstrated a significant interaction between HSS infusion and experimental group. When *a posteriori* analysis was performed after three-way ANOVA to compare peak ACTH responses to nitroprusside, the peak values measured after HSS infusion in the estradiol- and sham-treated fetuses (888 ± 450 and 404 ± 92 pg/ml, respectively) were significantly lower than peak values in each group after saline infusion (2239 ± 874 and 1045 ± 429 pg/ml, respectively). There was no significant difference between peak values after HSS- and saline- infusion in the androstenedione-treated fetuses (957 ± 99 vs. 1237 ± 311 pg/ml, respectively).

Nitroprusside infusion significantly increased fetal plasma cortisol concentrations in all groups except the estradiol-treated, saline-infused fetuses (Figure 4.6). Analysis of these data by two-way ANOVA revealed that the fetal plasma cortisol response to hypotension was not suppressed in any group. In both sham and androstenedione-treated fetuses, fetal plasma cortisol concentration was significantly higher in the HSS group than in the saline group. In the saline-infused estradiol-treated fetuses, fetal plasma cortisol

was already elevated as the result of the spontaneous elevation in fetal HPA axis activity prior to nitroprusside infusion. For this reason, the fetal plasma cortisol concentrations were not increased further in response to the nitroprusside infusion, and there was no apparent suppression of the fetal plasma cortisol response to nitroprusside after HSS infusion.

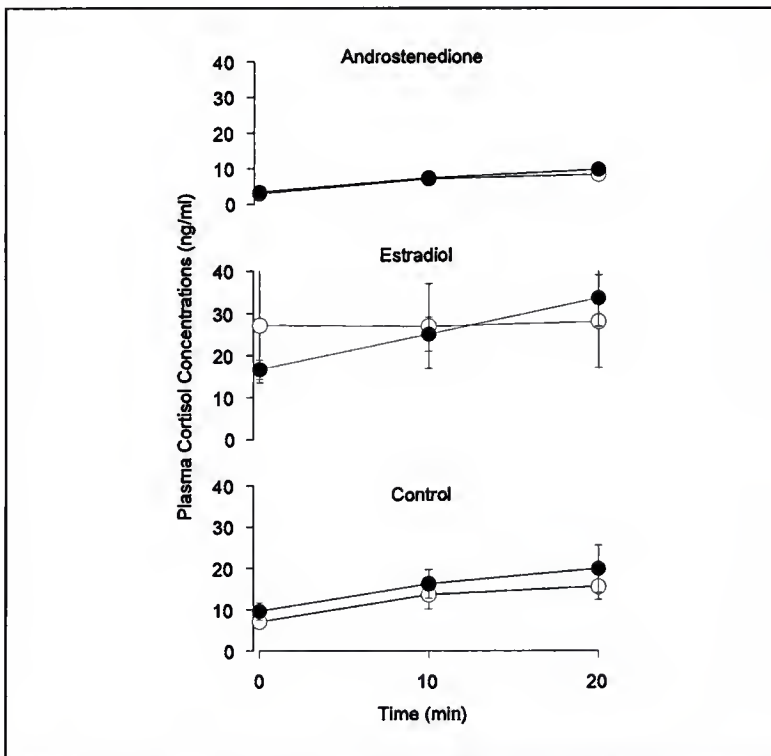


Figure 4.6. Fetal plasma cortisol responses to nitroprusside infusion in androstenedione, estradiol, and sham control fetuses following 2 hour saline (○) or HSS (●) infusion. Nitroprusside was infused between 0 and 10 minutes. Data are represented as mean $\pm$ SEM.

4.4 Discussion

The results of this study demonstrate that 1) estradiol augments fetal ACTH responses to hypotension; and 2) androstenedione decreases the efficacy of cortisol negative feedback inhibition of fetal plasma ACTH secretion. We propose that the increase in hypothalamic-pituitary-adrenal axis activity resulting from physiological increases in fetal plasma androgen and estrogen concentrations is an important component in the preparturient increase in fetal ACTH and cortisol secretion at the end of gestation in this species.

In the present experiments, the effect of exogenous androstenedione appeared to be different from the effect of the exogenous estradiol. Estradiol augmented the plasma ACTH response to hypotension, while androstenedione reduced cortisol negative feedback efficacy. It is remarkable that, even though estradiol doubled the fetal ACTH response to hypotension, HSS suppressed the fetal ACTH responses approximately 60% in both the estradiol- and sham-treated groups. This was consistent with the observation in adult animals that cortisol negative feedback suppression of ACTH responses to low- and moderate intensity-stimuli were essentially independent of stimulus intensity (Keller-Wood and Dallman, 1984). On the other hand, exogenous androstenedione completely eliminated cortisol negative feedback. We believe that the effect of androstenedione is similar to the spontaneous reduction in cortisol negative feedback efficacy observed at the end of gestation (Wood, 1988).

These results are consistent with the known actions of estrogens in adult animals. In adult rats, ovariectomy has been shown to decrease both basal and ether-stimulated plasma ACTH concentrations and to decrease pituitary responsiveness to stimulation by

hypothalamic extracts *in vitro* (Coyne and Kitay, 1969). Female rats have increased corticosterone responses to stress of exposure to ether vapors and have a greater adrenal responsiveness to exogenously administered ACTH when compared to male rats (Kitay, 1961). In ovariectomized rats, exogenously administered estradiol increased plasma ACTH bioactivity and adrenal responsiveness to ACTH (Kitay, 1963), and increased plasma ACTH and corticosterone responses to stress (Burgess and Handa, 1992; Viau and Meaney, 1991). However, our results differ from those of Burgess and Handa (1992) who reported that estradiol administration to ovariectomized rats impaired glucocorticoid negative feedback inhibition of ACTH secretion. We found that androgen, not estrogen, impaired glucocorticoid negative feedback efficacy in sheep fetus.

The effect of androgen on ovine fetal ACTH secretion also differed from that in the rat. Castration of male rats chronically increased both basal and stimulated plasma ACTH concentrations (Coyne and Kitay, 1971; Handa et al., 1994). Treatment of castrated rats with testosterone (Coyne and Kitay, 1971; Handa et al., 1994) or dihydrotestosterone (Handa et al., 1994) reversed this effect. It is therefore apparent that androgen is inhibitory to ACTH secretion in the male rat. The lack of effect of castration on hippocampal, hypothalamic, or pituitary corticosteroid receptors suggests gonadal steroids do not act through corticosteroid negative feedback in rats (Handa et al., 1994). We found that administration of androstenedione does not by itself alter basal or stimulated ovine fetal plasma ACTH, but did block corticosteroid negative feedback effects on ACTH. Whether this represents a species difference or, perhaps, a difference in experimental paradigm (intact fetal sheep exposed to both gonadal and placental steroids versus gonadectomized adult rats) cannot be addressed without further experiments.

HSS infusion had a tendency to increase mean arterial pressure compared with saline infusion. Excesses of glucocorticoids were shown to be associated with hypertension in man and in animals (Grunfeld, 1990). The HSS infusion used in these experiments utilized minimal concentrations, increasing plasma cortisol concentrations approximately 5 pg/ml in the androstenedione fetuses. In these fetuses, MAP was increased approximately 5 mmHg, which was statistically significant. However, in all experimental groups, there was no significant difference in the MAP drop, indicating a similar degree of hypotension created by the nitroprusside infusion.

The doses of estradiol and androstenedione used in the present experiments are physiological. The present results demonstrate a high sensitivity of the fetal hypothalamus-pituitary-adrenal axis to estradiol and androstenedione. The release of estradiol or androstenedione from implanted pellets (approximately 238 $\mu\text{g/day}$ or 9.9 $\mu\text{g/hr}$, as stated by Innovative Research of America, the supplier of the pellets) elevated plasma estradiol and androstenedione concentrations approximately 28 and 70 pg/ml, respectively. These concentrations were well within the range of endogenous concentrations reported at the end of gestation (Yu et al., 1983).

The apparent stimulation of the fetal HPA axis by estradiol increased fetal plasma ACTH and cortisol concentrations unrelated to the infusions of saline, HSS, or nitroprusside. Specifically, 3 of the 5 estradiol-treated fetuses displayed spontaneously increased fetal plasma ACTH during saline infusion (at -60 min: up to 1670, 2560, and 2742 pg/ml). The result of this spontaneous HPA activity was elevated mean plasma ACTH concentrations at time 0 in the saline-infused estradiol-treated fetuses compared to HSS-infused fetuses (707 ± 473 vs. 129 ± 49 pg/ml, respectively). We believe that

spontaneous HPA activity also complicated measurement of changes in fetal plasma cortisol in the HSS-infused group. We administered HSS at a rate of 0.5 $\mu\text{g}/\text{min}$ for 2 hours, a dose which completely suppressed fetal ACTH responses to the same dose of nitroprusside when infused for 5 hours (Wood, 1986). The expected increase in fetal plasma cortisol concentration during this infusion was small (Wood, 1986), but was completely obscured in the present experiments by the variable baseline in fetal cortisol and the relatively infrequent blood sampling.

We were unable to demonstrate statistically significant differences in the fetal plasma cortisol response to nitroprusside infusion. We believe that the fetal cortisol response to nitroprusside infusion in the present studies was limited by the maximal adrenal secretion rate of cortisol. The peak fetal plasma concentrations of cortisol measured in each group were consistent with known maximal fetal plasma cortisol concentrations in late-gestation fetuses (Rose et al., 1982).

A preparturient increase in maternal and fetal plasma estrogen concentration has been documented by a number of investigators, although the time course of this increase varies within studies. Maternal unconjugated estrogens have been reported to increase over the last 40 hours (Robertson and Smeaton, 1973) to 4 days (Bedford et al., 1972) with a very sharp increase immediately before parturition (Challis, 1971). In the fetus, a similar pattern has been reported for conjugated and unconjugated estrone (Nathanielsz et al., 1982); however, data in one study demonstrated a more gradual increase in fetal and amniotic estradiol over approximately 8 days with a rapid increase in fetal, maternal and amniotic concentrations in the final two days of fetal life (Challis and Patrick, 1981). Increased estrogens are also seen during dexamethasone-induced parturition (Kendall et

al., 1977). The fetuses in this study were subjected to 17β -estradiol chronically over the course of approximately two weeks, a pattern which does not faithfully mimic the pattern of plasma estradiol concentrations at term in intact fetal sheep. While the present data cannot be interpreted as evidence that fetal plasma estradiol represents a "trigger" to parturition, the results suggest that estrogens play a facilitatory role in HPA axis activation at the end of gestation by increasing the magnitude of the ACTH responses to stimuli.

We conclude that exogenous androstenedione and 17β -estradiol in physiological concentrations modulate plasma ACTH secretion in late-gestation fetal sheep. Estrogen increased plasma ACTH both during nitroprusside-induced hypotension and during apparently "basal" conditions. Androstenedione decreased the negative feedback action of cortisol on ACTH secretion (demonstrated by the lack of suppression of the ACTH response to nitroprusside-induced hypotension following the infusion of HSS). We therefore speculate that physiological increases in fetal plasma androgen and estrogen concentrations at the end of gestation are an important component of the mechanism which results in parturition in this species.

CHAPTER 5

ONTOGENY AND MOLECULAR WEIGHT OF IMMUNOREACTIVE ARGININE VASOPRESSIN AND CORTICOTROPIN-RELEASING FACTOR IN THE OVINE FETAL HYPOTHALAMUS

5.1 Introduction

In sheep, parturition is stimulated by an increase in the activity of the fetal hypothalamus-pituitary-adrenal (HPA) axis (Liggins et al., 1973; Challis and Brooks, 1989). This is observed as an increase in plasma concentrations of adrenocorticotropin hormone (ACTH) and cortisol in the fetus. Increased fetal plasma concentrations of cortisol induce the activity of cytochrome P450_{c17} (Anderson et al., 1975; Steele et al., 1976), an enzyme that catalyzes the synthesis of estrogens from progesterone. Near the end of gestation, fetal plasma ACTH secretion is further increased as cortisol negative feedback sensitivity decreases (Wood, 1988), thereby increasing fetal plasma cortisol concentrations and placental estrogen production. An increase in the rate of production of estrogens relative to progesterone increases uterine contractility and ultimately induces labor and delivery (Liggins, 1974).

Both corticotropin releasing factor (CRF) and arginine vasopressin (AVP) stimulate the release of ACTH from corticotrophs. In rats, CRF is more potent than AVP in stimulating ACTH secretion. In sheep, AVP is more potent (Familarì et al., 1989; Liu et al., 1990). In both species, CRF and AVP are synergistic when corticotrophs are exposed to both (Brooks and White, 1989). ACTH secretion *in vivo* is most likely the

result of combined CRF and AVP secretion. CRF- and AVP-containing neurons within the hypothalamus are thought to be the primary controllers of ACTH secretion. In rats, 50% of the AVP-positive parvocellular neurons of the SON and PVN also stain for CRF (Whitnal et al., 1987). AVP and CRF have been found in the same secretory vesicles in these neurons (Whitnal et al., 1985). AVP from magnocellular neurons of the PVN is thought to play a role in the stimulation of ACTH secretion, although this role is secondary to that of the parvocellular AVP (Holmes et al., 1986; Raff et al, 1985).

We hypothesized that hypothalamic content of immunoreactive CRF and/or AVP (iCRF and iAVP) increase at the end of gestation. We further hypothesized that the hypothalamic content of iCRF and iAVP is comprised of both processed and unprocessed forms of these hormones. The present experiments were designed to test these hypotheses.

5.2 Materials and Methods

Tissues. Experiments were performed using tissues collected from pregnant ewes of known gestational ages. Fetal hypothalami and pituitaries were harvested from healthy fetuses at various gestational ages (74-101 days, 125-129 days, 135-139 days, 141 days-term) and from 3- 4 week old healthy lambs as previously described. The tissues were stored at -20°C or -40°C until processed.

Tissues were later homogenized and processed as previously described. Protein concentrations of the homogenates were determined and homogenates were stored at -40°C until assayed or run on gels.

Hormone assays. Tissue content of iCRF and iAVP was determined by radioimmunoassay (RIA) of peptides in Laemmli buffer homogenates. Hypothalamic

tissues were diluted 1:100 with assay buffer and pituitary tissues were diluted 1:500-20,000. Tissue content is expressed as pg or ng hormone/mg protein. Descriptions of these radioimmunoassays were published previously (Keller-Wood and Wood, 1991, and Raff et al., 1991, respectively). After dilution, we found no interference of the Laemmli buffer with the binding of peptides to the antisera.

Sephadex chromatography. Three fetal hypothalami (ages 138, 138 and 143 days of gestation) were homogenized in CRF assay buffer (.05M phosphate, .01M EDTA, 0.1% v/v Triton, 0.1% w/v sodium azide) with 5% v/v glycerol, then centrifuged to remove the cellular debris. Samples were applied to a 49 x 1.5 cm Sephadex G75 column (Pharmacia, Uppsala, Sweden). The column was primed with 2 ml charcoal stripped sheep plasma, calibrated with molecular weight standards (Sigma), and equilibrated with CRF assay buffer. 100 μ l of the tissue homogenate or synthetic oCRF standard was added directly to the column in a mixture containing blue dextran and phenol red in CRF buffer with 5% v/v glycerol. Fractions were collected every 2 min at a flow rate of 600 μ l/min and frozen at -20° until assayed. Both iCRF and iAVP concentrations were measured from each fraction. To correct for interference by the CRF buffer system with the AVP antibody, the immunoreactive AVP was calculated from a standard curve prepared in CRF buffer and the assay was performed as described previously (Raff et al., 1991).

Western blotting. Western blots were performed as described previously. The protein was then transferred to nitrocellulose membranes and examined for the presence of iAVP and iCRF using the antibodies that were used in RIAs of these hormones. Blots were visualized as previously described and analyzed by densitometry. Antibody specificity was determined by preabsorption of the primary antibody with synthetic peptide and identification of non-specific banding patterns.

Statistical analysis. Initial analysis of the data for heteroscedasticity (distribution of error) revealed that they were not normally distributed. Overall comparison of iCRF and iAVP concentrations among the tested ages was performed using the Kruskal Wallis H test (one way analysis of variance on ranks). Multiple comparisons were tested using Dunn's test. Data are expressed as mean $\pm$ SEM. Significance was established at $p < .05$. Statistical procedures were accomplished using SigmaStat (Jandel Scientific, San Rafael, CA).

5.3 Results

iAVP. The ontogeny of pituitary immunoreactive AVP (iAVP) content is shown in Figure 5.1. There was no statistically significant effect of gestational age on pituitary AVP content within the range of ages tested ($H=7.62$, $df=4$, $p=0.10$). The fetal ontogeny of hypothalamic iAVP is shown in Figure 5.2. iAVP concentration in hypothalamic tissue increased significantly as a function of age ($H=13.6$, $df=4$, $p < 0.01$). The lowest iAVP concentration was measured in the 125-129 day fetal age group, and the highest in the 3-4 week old lambs (Fig. 5.2). The iAVP concentrations at these two ages were significantly different from each other when tested by Dunn's test ($p < 0.05$).

iCRF. The ontogeny of fetal hypothalamic immunoreactive CRF (iCRF) concentration is shown in Figure 5.3. Hypothalamic iCRF concentration increased significantly as a function of age ($H=16.9$, $df=3$, $p < 0.001$). The increase in hypothalamic iCRF concentration appeared more gradual than the increase in hypothalamic iAVP concentration. Thus, the concentrations in the term fetuses and the 135-139 day fetuses were significantly greater than in the 74-101 day fetuses.

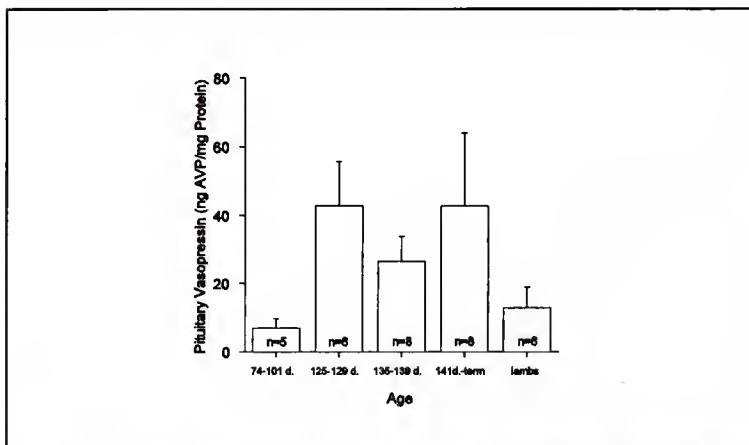


Figure 5.1. Pituitary content of immunoreactive arginine vasopressin. Gestational age did not significantly effect pituitary content of iAVP within the range of ages tested ($H=7.62$, $df=4$, $p=0.10$).

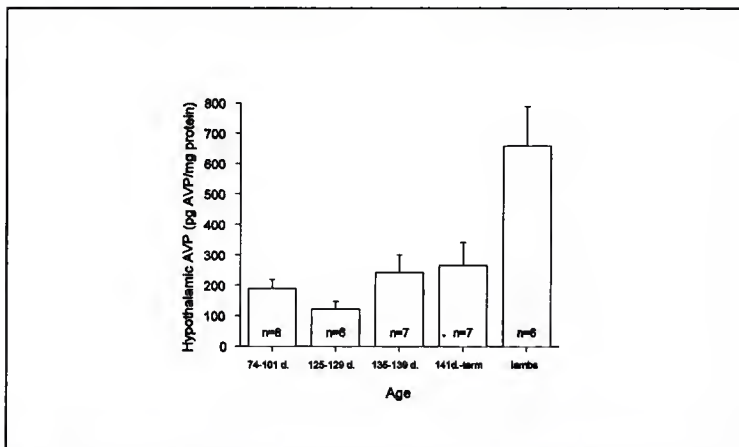


Figure 5.2. Hypothalamic content of immunoreactive AVP. Tissue content of iAVP in the fetal hypothalamus increased significantly as a function of age ($H=13.6$, $df=4$, $p<0.01$).

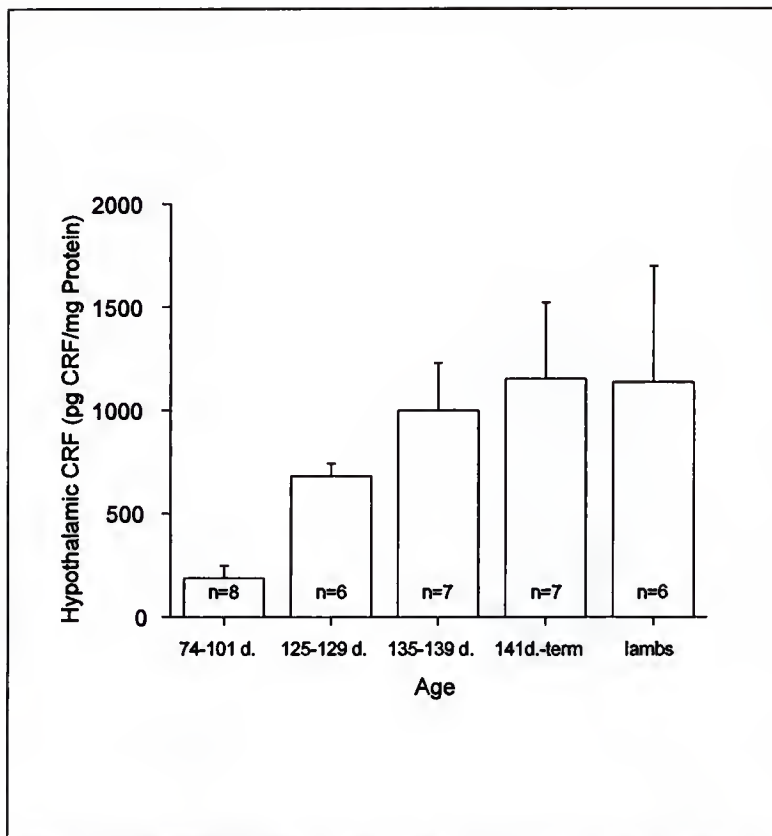


Figure 5.3. Hypothalamic content of immunoreactive CRF (iCRF). Tissue content of iCRF in the fetal hypothalamus increased significantly as a function of age ($H=16.9$, $df=4$, $P<0.001$).

Molecular weight analysis. The results of size exclusion chromatography (Sephadex G75) for iCRF are shown in Figure 5.4. iCRF isolated from fetal hypothalamus (fig. 5.4, top) eluted earlier than the synthetic ovine CRF peptide (fig. 5.4, bottom),

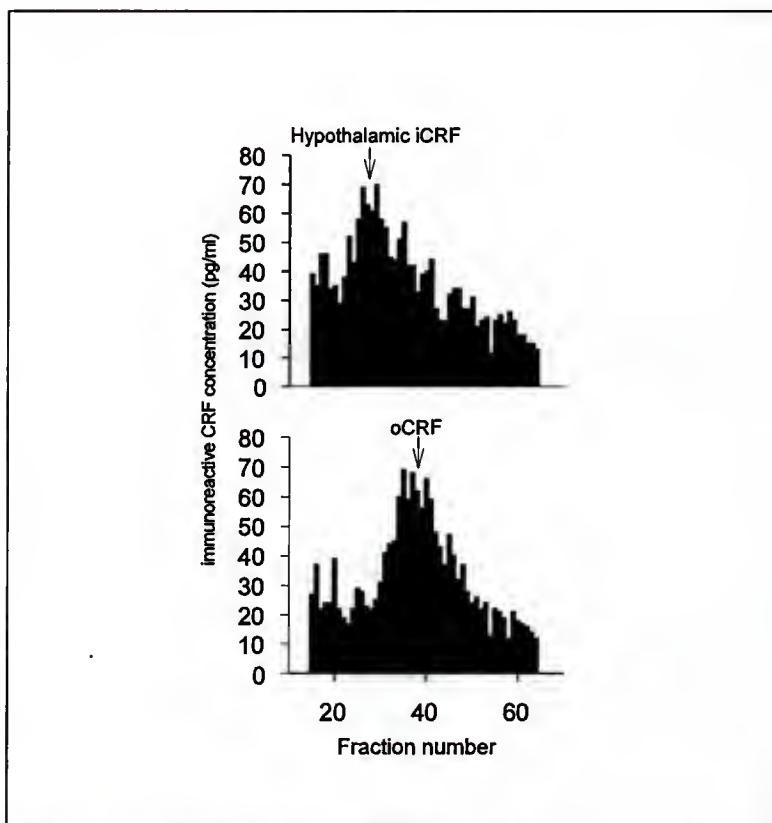


Figure 5.4. Hypothalamic iCRF eluted faster than synthetic oCRF. This indicates a larger molecular weight form than the synthetic CRF.

demonstrating that the peptide found in this tissue had a higher molecular weight than fully processed CRF. Hypothalamic tissue analyzed by Western blot revealed a band which migrated with the 21.5 kD molecular weight marker (fig. 5.5), a weight which is consistent with the molecular weight of pro-CRF, and another band with a molecular

weight which was approximately 45 kD. These bands were specific for iCRF since preabsorption of the antiserum with synthetic oCRF reduced the intensity of the staining.

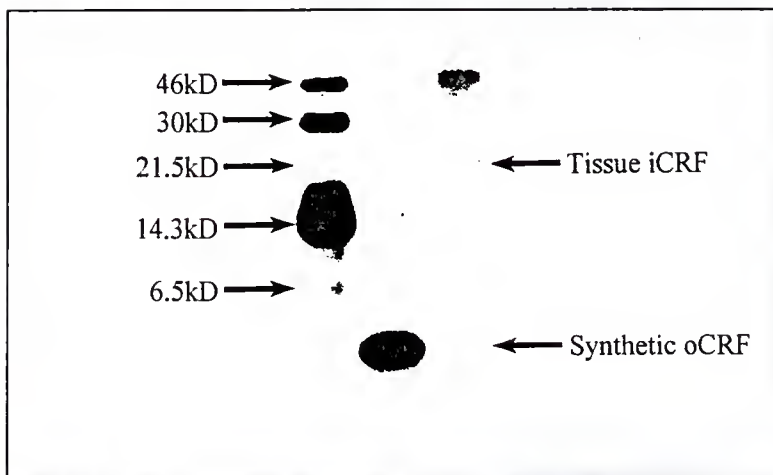


Figure 5.5. Western blot analysis of hypothalamus homogenate. Bands specific for iCRF are denoted by arrows.

iAVP was eluted from the Sephadex column in two molecular weight forms (fig. 5.6). The major peak of iAVP is consistent with fully processed AVP (~1 kD). A second major peak was found in the void volume, suggesting the presence of a high molecular weight moiety. The high molecular weight forms of iAVP were re-examined using Western Blot. A band was clearly visible with a molecular weight of approximately 20 kD and another band was visible with a molecular weight of approximately 40 kD (fig. 5.7). These bands represent specific staining for iAVP, since the intensity of staining is reduced after preabsorption of the antiserum with synthetic peptide.

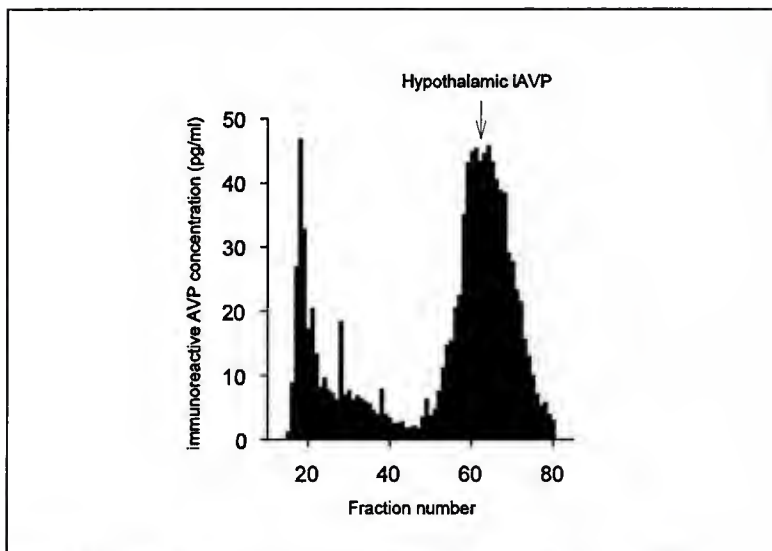


Figure 5.6. Elution profile of iAVP in hypothalamic homogenate.

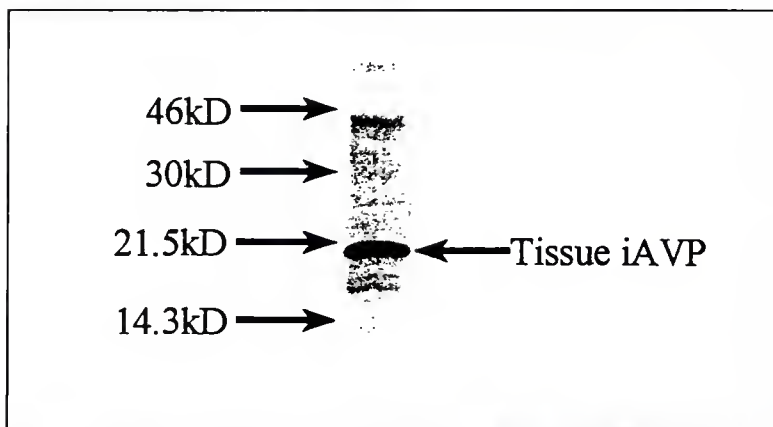


Figure 5.7. Western blot analysis of hypothalamus homogenate. Bands specific for iAVP are denoted by arrows.

5. 4 Discussion

The results of this study indicate that concentrations of ACTH releasing hormones in hypothalamic tissue changed with time throughout development. Hypothalamic iAVP did not increase significantly until postnatal life; however, the highest concentrations of hypothalamic iCRF were measured at the end of gestation. The measured increases in the concentrations of these hormones demonstrate that maturation of the hypothalamus-pituitary-adrenal axis includes a maturation of the secretory capacity of hypothalamic neurons. The pattern of iCRF relative to that for iAVP suggests that changes in iCRF secretory capacity at the end of gestation might be an important factor in the stimulation of the preparturient fetal ACTH surge.

The ontogeny of iCRF and iAVP measured in the present study was remarkably similar to that reported by Brieu et al. (1989), although we were unable to demonstrate any decrease in iCRF or iAVP in animals near term gestation (141 days to term, Figures 5.2 and 5.3). Our demonstration of increased iCRF as a function of fetal gestational age was also similar to data reported by Watabe et al. (1991) and Currie and Brooks (1992), as stated previously. We speculate that this developmental increase is important to increased plasma ACTH secretion in fetal sheep observed near the end of gestation.

Watabe, et al. (1991) examined hypothalamic iCRF in fetal sheep ranging in age from 48-140 days of gestation. Currie and Brooks (1992) examined hypothalamic iCRF in fetal sheep ranging in age from 70-130 days of gestation. The results of our present study agree with the results of the previous studies in that we demonstrated an increase in fetal hypothalamic iCRF concentration at the end of gestation. The results of our study differed, however, in that we found a high molecular weight form of iCRF in fetal

hypothalamus. In the Watabe study, only one molecular weight species of iCRF was identified in hypothalamic extracts (Watabe et al., 1991). We detected iCRF with a higher molecular weight than fully processed CRF using Sephadex gel chromatography. The results of Western blot analysis demonstrated one molecular form of iCRF consistent with the known molecular weight of pro-CRF (Lauber et al., 1984) and another with molecular weight consistent with a dimer of pro-CRF. We believe that there are two likely explanations for the difference between our results and those reported by other investigators. First, differences might be due to the binding characteristics of the antibodies used in the studies. Our anti-CRF antibody recognized the N-terminal region of ovine CRF (Keller-Wood and Wood, 1991) while Watabe, et al. used an antibody that recognized the C-terminal end of rat CRF. Second, we quantified immunoreactive peptides in tissue which was homogenized directly in a reducing buffer. The previous studies quantified the peptides after acid extraction, a protocol which could have been confounded by poor recovery of or proteolysis of the unprocessed forms of the peptides. Experiments with bovine hypothalamic CRF, for example, have shown that lyophilization, boiling for 15 min, or boiling at a low pH shifted CRF immunoreactivity and/or bioactivity to a higher fraction, suggesting proteolytic cleavage of the peptide (Yasuda and Yasuda, 1985a; Yasuda and Greer, 1978). The evidence for large molecular weight forms of iCRF in the present study is consistent with data from human fetal (Ackland et al., 1986), human adult (Suda et al., 1984), and rat (Yasuda and Yasuda, 1985b) hypothalami.

Currie and Brooks (1992) found one molecular weight form of iAVP which corresponded to synthetic AVP in their hypothalamic extracts. This contrasts with the results of the present study, and with the known processing of AVP in other species.

Sephadex chromatography demonstrated a form of iAVP with a molecular weight consistent with fully processed AVP (fig. 5.5a), and a larger molecular weight form. Western Blot analysis demonstrated one form with a molecular weight (~20 kD) consistent with pro-AVP (Russell, et al., 1979), and another form with a molecular weight of approximately 40 kD. It is likely that the 40 kD form is a dimer of pro-AVP. The molecular weight of pro-AVP has been estimated to be 17,310 kD in cows (Land et al., 1982) and 20,000 kD in rats (Russell et al., 1979). The presence of pro-AVP in hypothalamic tissue is consistent with the known processing of AVP in rats (Russell et al., 1979). We believe that Currie and Brooks measured only the fully processed AVP because of the binding characteristics of the anti-AVP antiserum used in their study (ie., it is possible that their antiserum did not cross-react with pro-AVP). It is also possible that processed and unprocessed forms of AVP were differentially recovered in the hydrochloric and acetic acid extraction procedure used in that study.

The observation that CRH mRNA in the hypothalamus is present and increases as a function of gestational age supports our results of CRF protein content (Matthews and Challis, 1995). In examining the hypothalamic AVP tissue content, we did not differentiate between magnocellular or parvocellular AVP. Matthews and Challis (1995) found that magnocellular AVP mRNA increased in late gestation. Parvocellular AVP mRNA did not change at this time, but the two increased in the newborn. These results are similar to our study in which we did not detect a significant increase in hypothalamic iAVP until after birth. In work performed by Keiger, et al., CRF message and peptide were measured in the fetal ovine hypothalamus over 4 gestational age ranges (95-106, 120-123, 128-138, and 140-142 days). CRF mRNA levels in the 140-142 day group were

significantly different than the other groups analyzed. However, the CRF peptide concentrations in the 140-142 day group were significantly greater than only the 95-106 and 128-138 day groups, showing a possible dissociation between message and protein levels.

In summary, we have demonstrated that 1) fetal hypothalamus contains precursor proteins for AVP and CRF; and 2) that hypothalamic iAVP and iCRF increases as a function of fetal and neonatal developmental age when measured by radioimmunoassay. An increase in tissue stores of ACTH releasing factors may reflect increased synthesis and secretion of these hormones and therefore an increased stimulus to ACTH secretion. The increased tissue stores of releasing hormones might complement the decrease in cortisol negative feedback inhibition of ACTH secretion that occurs late in gestation during the preparturient rise in fetal ACTH secretion. Therefore, this increase in iAVP and iCRF might be an important component of the increase in hypothalamus-pituitary-adrenal activity that initiates parturition in this species.

CHAPTER 6

ONTOGENY OF PROOPIOMELANOCORTIN POSTTRANSLATIONAL PROCESSING IN THE OVINE FETAL PITUITARY

6.1 Introduction

In the sheep, parturition is stimulated by an increase in the activity of the fetal hypothalamus-pituitary-adrenal (HPA) axis (Liggins et al., 1973; Challis and Brooks, 1989). In late gestation there is an increase in fetal HPA axis activity which increases plasma adrenocorticotropin (ACTH) and cortisol concentrations. Cortisol acts at the placenta to increase production of estrogens, which increase uterine contractility and ultimately leads to labor and delivery (Liggins, 1974).

ACTH is initially synthesized as pro-opiomelanocortin (POMC), a precursor for ACTH as well as β -endorphin, β -lipotropin, and α -melanocyte-stimulating hormone (Eipper and Mains, 1982). After synthesis, POMC, a 32 kilodalton (kD) peptide, is cleaved twice in order to release ACTH (Figure 6.1; Schnabel et al., 1989). The first processing step yields a 22 kD fragment (so-called "pro-ACTH") which contains the sequence for ACTH. The second step yields fully processed ACTH. Because POMC, 22kD fragment, and fully processed ACTH (ACTH₁₋₃₉) all contain the ACTH epitope, anti-ACTH antisera generally recognize all three forms of ACTH.

Evidence from several laboratories has demonstrated that fully processed ACTH₁₋₃₉ stimulates fetal adrenal secretion of cortisol; in addition, however, the larger molecular

weight forms of immunoreactive ACTH (iACTH) appear to antagonize the effect of ACTH₁₋₃₉ (Jones and Roebuck, 1980; Roebuck and Jones, 1980). This differential effect of fully processed ACTH, and unprocessed and partially processed POMC produces changes in the ratio of biologically active ACTH (bACTH) to iACTH in various conditions.

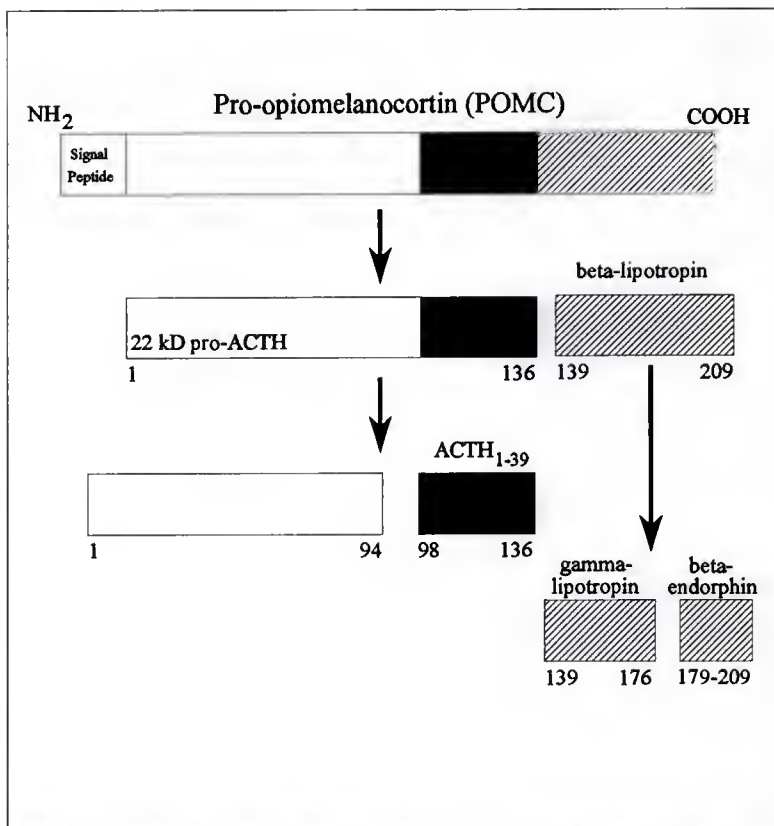


Figure 6.1. Diagram of the stepwise processing of ACTH from POMC, including other major products of POMC processing. Figure adapted from Schnabel et al. (1989).

In ovine fetal plasma, the concentration of fully processed ACTH increases relative to that of the larger molecular weight forms (Thorburn et al., 1991). The present study was designed to test the hypothesis whether the concentration of fully processed ACTH increases in fetal pituitary relative to that of either POMC or pro-ACTH with development. We also designed these experiments to test the hypothesis that administration of exogenous estrogens and androgens increase the pituitary concentration of fully processed ACTH observed in mature fetuses.

6.2 Materials and Methods

Tissues. Experiments were performed using tissues collected as previously described from healthy fetuses at various gestational ages (74-101 days, 125-129 days, 135-139 days, 141 days-term) and from 3-4 week old healthy lambs. Fetal tissues were rapidly collected, quick-frozen, and stored at -20°C . Tissues were processed as previously described. Protein concentrations of the homogenates were determined and homogenates were stored at -40°C until assayed or run on gels.

Steroid treated fetuses. Fetuses were treated with 21 day, 5 mg 17β -estradiol or androstenedione pellets implanted in the area of the gluteus medius on about day 120 of gestation (approximately $238\text{ }\mu\text{g}$ per day). We had previously reported that implantation of estradiol pellets increased fetal plasma estradiol concentration to $39.1\pm 11.1\text{ pg/ml}$ compared to control fetuses, in which fetal plasma estradiol concentration was $11.4\pm 3.9\text{ pg/ml}$ (Chapter 4). Implantation of androstenedione pellets increased fetal plasma androstenedione concentrations to $273\pm 20\text{ pg/ml}$ compared to control fetuses in which fetal plasma androstenedione concentration was $203\pm 10\text{ pg/ml}$ (refer to Chapter 4). Fetal pituitaries were harvested one to two weeks after pellet implantation.

Hormone assays. Tissue content of iACTH was determined by radioimmunoassay (RIA) of peptides in Laemmli buffer homogenates. Pituitary homogenates were diluted 1:10,000 with assay buffer and tissue content was expressed as ng hormone/mg protein. The ACTH RIA was performed as previously described (Bell et al., 1991). After dilution with assay buffer, we found no interference of the Laemmli buffer with the binding of ACTH to the antisera. However, the concentration of ACTH as determined by the immunoreactivity did not dilute parallel to the standard curve, so the assay was performed with all the samples at the same dilution.

Western blotting. Western blots were performed as previously described on 16.5% Tris-tricine precast polyacrylamide gels. An equal amount of protein (20µg) was loaded in each lane. The protein was then transferred to PVDF membranes and probed for iACTH using the same antibody that was used in the RIA (dilution of 1:13,000). Blots were visualized by a Western blot chemiluminescence reagent and analyzed by densitometry. Antibody specificity for iACTH staining was determined by preabsorption of the primary antibody with 100µg synthetic ACTH per blot and identification of non-specific banding patterns. Synthetic ACTH was also run on each gel as a marker for ACTH₁₋₃₉.

Statistical analysis. Statistical procedures were accomplished using SigmaStat (Jandel Scientific, San Rafael, CA). Differences between pairs of means were assessed using Student's t-test. Ontogenetic changes in measured or calculated variables were assessed using simple linear regression or multiple linear regression. For the purpose of performing regression analysis, we estimated the ages of the lambs as 175 days. In each statistical test, the null hypothesis was rejected when $p < 0.05$.

6.3 Results

There was a variable, yet statistically significant ($p < 0.05$), relationship between pituitary iACTH concentration and developmental age (Figure 6.2). The age-related changes in pituitary iACTH concentration were further analyzed by Western Blot technology to identify possible changes in the relative concentrations of the different molecular species which comprise iACTH.

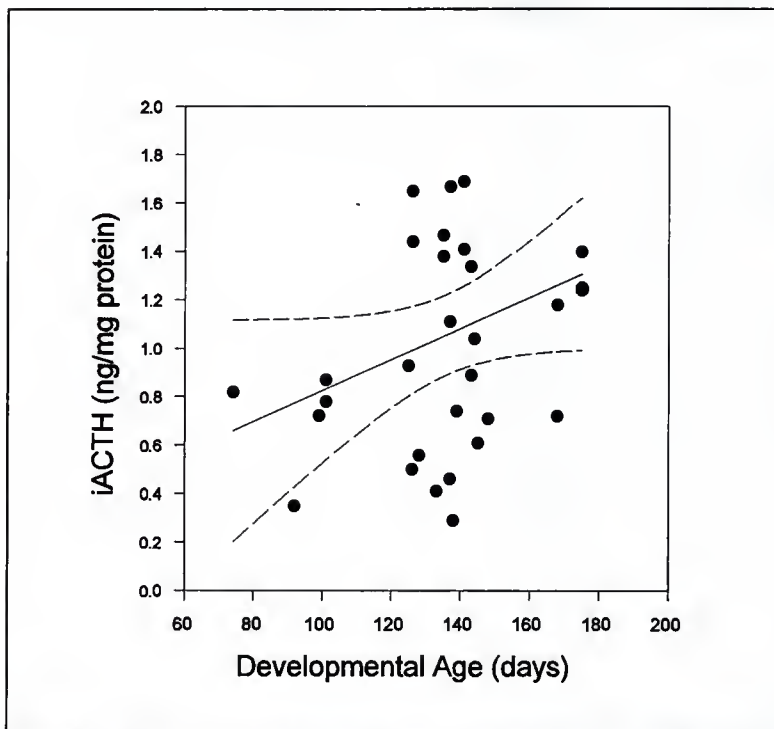


Figure 6.2. Pituitary immunoreactive ACTH content (ng/mg protein) as a function of developmental age. There was a significant relationship between pituitary iACTH and developmental age ($p < 0.05$). Data are represented as mean (solid line) with a 95% confidence interval (dashed lines).

As shown in Figure 6.3, we were able to separate the three major molecular forms of iACTH: POMC, 22 kD fragment, and fully processed ACTH. The staining for these peptides was immunologically specific, since preabsorption of the antiserum with synthetic human ACTH₁₋₃₉ decreased the intensity of the staining of all three bands (Figure 6.3).

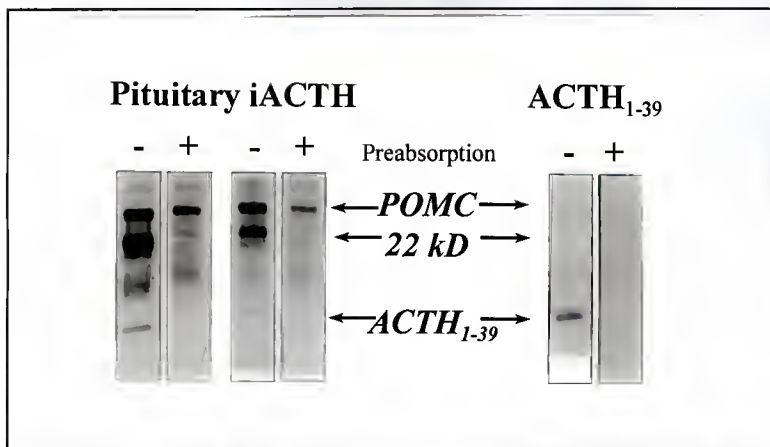


Figure 6.3. Western blot analysis of pituitary homogenate iACTH. Bands specific for iACTH are denoted by arrows.

Although each gel contained tissue samples representing a wide range of developmental ages, statistical analysis of the data was complicated by the variation in the intensity of staining and variation in the exposure of the film during the chemiluminescence reaction. We corrected for this variation by analyzing the optical density data for each molecular form of iACTH (POMC, pro-ACTH, and ACTH₁₋₃₉) by multiple regression analysis, using developmental age and gel number as independent variables. This analysis revealed a statistically significant ($p < 0.05$) influence of age on the intensity of staining in the band representing fully processed ACTH (Figure 6.4), but no statistically significant

changes were shown in the other two bands (data not shown). In each analysis, the variation attributable to the variations in gel density was statistically significant ($p < 0.001$).

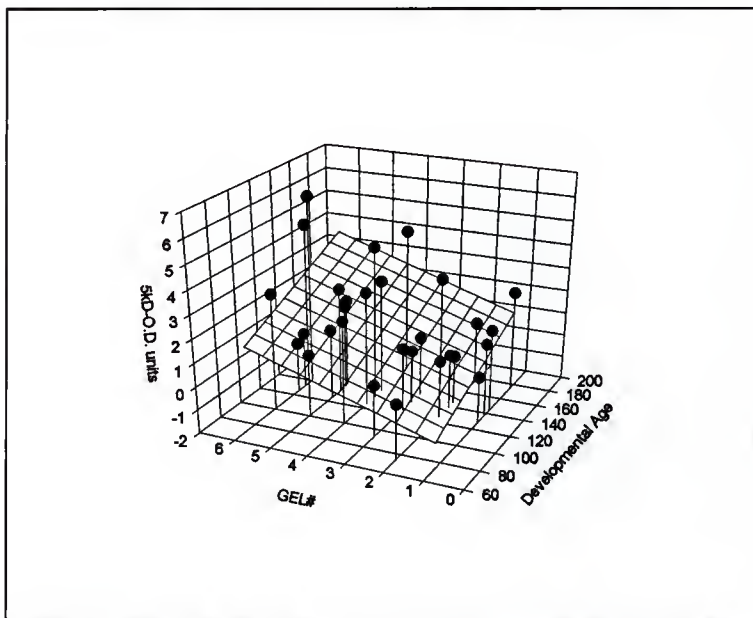


Figure 6.4. Multiple regression analysis of 5kD ACTH optical density (O.D.). Staining of ACTH₁₋₃₉ increased as a function of developmental age with respect to gel number ($p < 0.05$). There was no relationship between POMC and pro-ACTH staining with age (data not shown).

To obtain an index of the changes in fully processed ACTH relative to the abundance of 22 kD fragment and POMC, we calculated an index of the percentage of iACTH which is fully processed to ACTH₁₋₃₉. This index is the ratio of the optical density in the band containing fully processed ACTH₁₋₃₉ and the sum of the optical density in the other bands. Analysis of this ratio by linear regression analysis revealed a statistically significant increase as a function of developmental age (Figure 6.5). As can be seen by

comparison of Figures 6.4 and 6.5, calculation of this ratio eliminated the need to analyze by multiple linear regression analysis, since variations in image density were normalized.

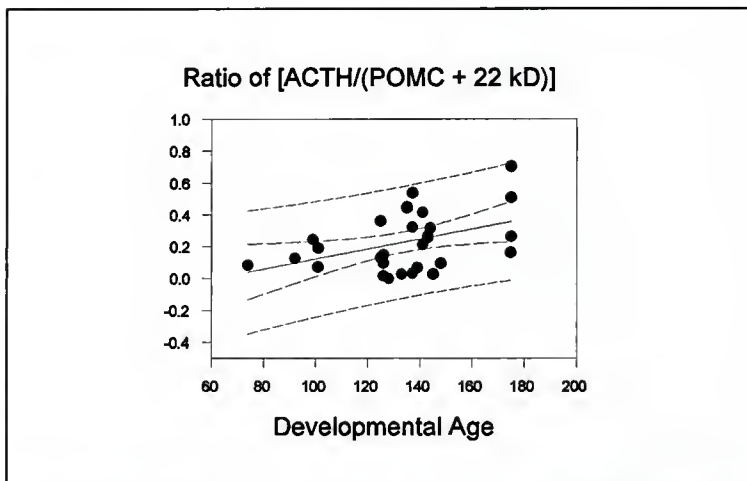


Figure 6.5. Linear regression analysis of the ratio of ACTH₁₋₃₉ and the sum of POMC and pro-ACTH.

As shown in Table 6.1, treatment of fetuses with estradiol or androstenedione chronically did not alter the concentration of iACTH in the fetal pituitary (measured by RIA). The processing of iACTH was studied in a subset of these steroid-treated animals using Western blot analysis. The index of processing, calculated as shown in Figure 6.5, was reduced during estradiol treatment but not by androstenedione treatment (calculations not shown). In 4 estradiol-treated fetuses compared to 7 age-matched control fetuses, the calculated index was $51 \pm 18\%$ of age-matched control fetuses. In 5 androstenedione-treated fetuses compared to 8 age-matched control fetuses, the calculated index was $159 \pm 85\%$ of the age-matched control fetuses.

Table 6.1. Fetal pituitary iACTH concentration in estradiol-treated, androstenedione-treated, and age-matched control fetuses.

Experimental Group	# of Fetuses	Age of Fetuses	iACTH (ng/mg protein)	Ratio of [ACTH / (POMC+22 kD)]
estradiol-treated	4	138 ± 0.9	1.14 ± 0.17	0.12 ± 0.05
control fetuses	7	139 ± 0.9	1.13 ± 0.14	0.27 ± 0.08
androstenedione-treated	5	128 ± 0.5	1.31 ± 0.11	0.22 ± 0.10
control fetuses	8	127 ± 1.1	1.10 ± 0.15	0.13 ± 0.05

6.4 Discussion

The results of this study demonstrate that the steady state concentration of iACTH in ovine fetal pituitary tissue was relatively constant throughout the last half of gestation, but that fully processed ACTH represented a significantly greater proportion of the total pituitary iACTH in late-gestation fetuses and lambs. Despite obvious variability in the data, there was a significant difference in the concentration of iACTH in the pituitary as a function of developmental age. However, chronic treatment of the fetuses with estradiol reduced the proportion of iACTH which is processed.

In sheep, the fetal hypothalamus-pituitary-adrenal axis acts as a "trigger" which initiates parturition. Late in gestation (approximately 120 days or approximately 80% of full-term gestation), plasma ACTH concentrations begin to increase. Fetal plasma ACTH and cortisol concentrations slowly increase until approximately 140 days (approximately 95%) gestation. At this time, cortisol negative-feedback efficacy decreases and cortisol is no longer able to "turn off" ACTH secretion from the pituitary. We believe that simultaneous with the decrease in cortisol negative feedback sensitivity is the onset of a

stimulation, or "drive", to ACTH secretion. Together, these changes in drive and feedback sensitivity increase fetal plasma concentrations of both ACTH and cortisol.

Both fetal and adult ovine corticotrophs secrete a mixture of fully processed ACTH_{1-39} and larger forms of iACTH. It has been proposed by other investigators that the relative proportions of processed and unprocessed forms of iACTH are important variables in determining adrenal responsiveness to circulating ACTH. It has been shown that high molecular weight precursors of ACTH inhibit the response to ACTH_{1-39} by fetal sheep adrenal cells (Jones and Roebuck, 1980; Roebuck and Jones, 1980). POMC is somewhat less effective than 22 kD iACTH, but both are effective inhibitors of ACTH action. Schwartz and colleagues have demonstrated that the 22 kD fragment inhibited the fetal adrenal response to ACTH_{1-39} in a 2.3 molar excess, while POMC inhibited the response in a 26 molar excess (1995). This interaction was not demonstrable in experiments using adrenal cortical cells from adult sheep (Schwartz et al., 1995). In the perfused fetal rat adrenal cells, POMC did not block the action of 4.5 kD ACTH. In fact, both ACTH_{1-39} and 22 kD fragment were steroidogenic, and the adrenal response to these peptides increased with fetal gestational age (Chatelain and Cheong, 1987).

The changing proportions of processed and unprocessed forms of iACTH in the ovine fetal pituitary probably explains the developmental changes in the so-called bioactive-to-immunoreactive ACTH ratio, or b/I ratio, during stimulation in fetal sheep. In the plasma of unstimulated fetal sheep, the b/I ratio for ACTH is low (Castro et al., 1993). In other words, the plasma concentration of ACTH measured by bioassay is low when compared to the plasma concentration of ACTH measured by RIA. When subjected to hemorrhage, the b/I ratio increases (Castro et al., 1993), probably reflecting

the secretion of ACTH₁₋₃₉ from the fetal pituitary. However, the b/I ratio during stimulation changes as a function of fetal gestational age (Castro et al., 1992). That is, the b/I ratio in plasma samples from stimulated fetuses is higher late in gestation compared with stimulated fetuses which are younger and less well developed (Castro et al., 1992). Changes in POMC processing are reflected in the molecular forms circulating in plasma from unstimulated fetuses. Hollingworth and Thorburn have demonstrated that the proportion of iACTH in fetal plasma which is fully processed increases late in gestation (Thorburn et al., 1991).

We (refer to Chapter 5) and others (Brieu et al., 1989; Watabe et al., 1991; Currie and Brooks, 1992) have reported developmental increases in ovine fetal hypothalamic concentrations of ovine fetal hypothalamic concentrations of ovine corticotropin releasing factor (oCRF) and arginine vasopressin (AVP), the two hypophysiotropic releasing factors for ACTH in the sheep. The ontogenetic changes in hypothalamic releasing factor concentrations complement the ontogenetic changes in pituitary ACTH concentration and processing reported in this study. Together these changes would support an increase in the activity of the hypothalamus-pituitary-adrenal axis as the fetus matures. In other words, these changes in tissue peptide concentrations most likely create a greater reserve for activity of the HPA axis. This reserve might be important during the preparturient surge in activity which precipitates parturition. The reserve could also be important during the neonatal period in which HPA axis responses to stress are important for survival.

We tested the effect of estradiol or androstenedione treatment on fetal pituitary iACTH concentration and on POMC processing to test the hypothesis that either steroid

would produce changes in the measured or calculated variables similar to the spontaneous developmental changes. We found that estradiol produced changes in POMC processing which were opposite those observed endogenously. Therefore, it is unlikely that increases in fetal plasma estrogen concentrations constitute the stimulus to increased POMC synthesis and processing. We have reported that chronic estradiol treatment increases both basal- and stimulated- fetal plasma ACTH concentrations (refer to Chapter 4). We have also reported that chronic androstenedione administration dramatically reduces the sensitivity of cortisol negative feedback inhibition of fetal ACTH secretion. The decrease in pituitary POMC processing produced by estradiol might simply reflect stimulation of pituitary secretion (increased secretion at the expense of intracellular stores). If so, the developmental increase in iACTH and POMC processing which occurs in the absence of exogenous steroid must be dependent upon another stimulus (ie., increased hypothalamic oCRF or AVP secretion).

Understanding changes in fetal pituitary posttranslational processing of POMC into the different molecular forms of iACTH are likely to be important in completely understanding the mechanism of parturition. However, it is important to recognize that the pituitary is not the only potential source of iACTH in fetal plasma. It has been demonstrated that the fetal lung contains iACTH (Cudd et al., 1993). When tested by Western blot analysis, pulmonary tissue contained only large molecular weight forms of iACTH, but not any measurable amounts of ACTH₁₋₃₉ (Cudd et al., 1993). *In vivo*, the lung secreted iACTH under both basal (Cudd and Wood, 1995) and highly stimulated (Cudd et al., 1993) conditions. In addition to secreting iACTH, the lung clears approximately 50% of the ACTH in the pulmonary arterial plasma (Cudd and Wood,

1995). While we do not know the molecular form of iACTH secreted by the lung, it is estimated that a significant proportion of the iACTH measured in the plasma of unstimulated fetal sheep could potentially originate in the fetal lung (Cudd and Wood, 1995). This suggests that, in addition to developmental changes in pituitary processing, developmental changes in pulmonary POMC biosynthesis and processing might be important in the timing of parturition.

In summary, we have demonstrated that the concentration of iACTH increases as a function of fetal gestational age in ovine fetal pituitary, and that the proportion of the total immunoreactivity which is fully processed to ACTH_{1,39} also increases. Estradiol pretreatment of the fetuses significantly decreases the proportion which is fully processed, an action which might be the result of estrogen-induced stimulation of fetal ACTH secretion.

CHAPTER 7

DEVELOPMENTAL CHANGES AND MOLECULAR WEIGHT OF IMMUNOREACTIVE GLUCOCORTICOID RECEPTOR PROTEIN IN THE OVINE FETAL HYPOTHALAMUS AND PITUITARY

7.1 Introduction

The glucocorticoid receptor (GR) belongs to a superfamily of steroid and thyroid hormone receptors that are ligand-dependent, nuclear transcription factors (Evans, 1988). The DNA sequence for the human GR has been identified (Hollenberg et al., 1985) as has a partial sequence for ovine GR (Yang et al., 1991), which has 92% identity to bases 143-453 of the human GR. Based on the deduced amino acid sequence, a number of new antibodies have been produced to peptide sequences in the amino-terminal end of the receptor. Based on the deduced amino-acid sequence, a number of new antibodies have been produced to peptide sequences in the amino-terminal end of the receptor. These antibodies recognize multiple forms of the receptor: unactivated receptor, activated DNA-binding receptor, and unliganded receptor. Previously available antibodies only recognized activated DNA-binding forms of the receptor protein, possibly due to an interaction of the antibody with an epitope of the receptor protein exposed after ligand binding. Cidlowski et al. (1990) produced two rabbit anti-human polyclonal antibodies (#57 and #59) using peptide sequences that are independent of the steroid and DNA binding domains as well as receptor oligomeric state. The binding characteristics of the antibody was determined using Hela cell cultures and

immunocytochemistry (McGimsey et al., 1991; Burnstein et al., 1991; Burnstein et al., 1990). In these systems, antibody #57 recognized a 97 kilodalton protein when analyzed by Western blot gel electrophoresis. The purpose of the present study was to use this GR antibody in a tissue homogenate preparation for identification of ovine immunoreactive GR protein in the fetal hypothalamus and pituitary.

In the sheep, parturition is stimulated by an increase in the activity of the fetal hypothalamus-pituitary-adrenal (HPA) axis (Liggins et al., 1973; Challis and Brooks, 1989). Prior to spontaneous parturition, plasma concentrations of adrenocorticotropin hormone (ACTH) and cortisol increase in the fetus. Near the end of gestation, fetal plasma ACTH secretion is further increased as cortisol negative feedback sensitivity decreases (Wood, 1988), thereby further increasing fetal plasma cortisol concentrations. Cortisol increases placental conversion of estrogens from progesterone, increasing uterine contractility, and initiating labor and parturition (Liggins, 1974). We hypothesized that the decrease in cortisol negative feedback efficacy that is seen late in gestation may be due to a reduction in glucocorticoid receptors in the fetal hypothalamus or pituitary.

7.2 Materials and Methods

Tissues. Experiments were performed using tissues collected from pregnant ewes of known gestational ages. Fetal hypothalami and pituitaries were harvested from healthy fetuses at various gestational ages (74-101 days, 125-129 days, 135-139 days, 141 days-term) and from 3- 4 week old healthy lambs. Fetal tissues were rapidly collected and snap-frozen using a dry ice-acetone bath. The tissues were stored at -20°C or -40°C until processed. Tissues were later homogenized in reducing buffer (Laemmli, 1970) and prepared as

previously described. Protein concentrations of the homogenates were determined and samples were stored at -40°C until analyzed by electrophoresis.

Steroid-treated fetuses. Fetuses were treated with 5 mg 17β -estradiol, androstenedione, or tamoxifen (estrogen antagonist) pellets designed to release over 21 days, implanted in the area of the gluteus medius on about day 120 of gestation (release rate of approximately 238 μg per day). Estradiol-, androstenedione- and tamoxifen-treated fetuses were sacrificed at 138 ± 1 ($n=4$), 128 ± 1 ($n=8$), and 128 ± 1 ($n=5$) days gestation, respectively. The hypothalamus from one androstenedione-treated fetus and the pituitary from one tamoxifen-treated fetus were not used because of errors in tissue preparation or collection. Age-matched controls for these groups consisted of fetuses which were 128 ± 1 ($n=14$) days gestation for the androstenedione- and tamoxifen-treated fetuses and 140 ± 1 ($n=13$) days gestation for the estradiol-treated fetuses. In other experiments, we found that implantation of estradiol pellets increased fetal plasma estradiol concentration from 11.4 ± 3.9 ($n=12$) to 39.1 ± 11.1 ($n=7$) pg/ml (Chapter 4). Implantation of androstenedione pellets increased fetal plasma androstenedione concentrations to 273 ± 20 ($n=8$) to control fetuses 203 ± 10 ($n=9$) pg/ml (Chapter 4). Fetal hypothalami and pituitaries were harvested one to two weeks after pellet implantation.

Western blotting. Western blot analyses were performed as described previously on 10% precast polyacrylamide gels. An equal amount of protein ($40\mu\text{g}$) was loaded in each lane. The protein was then transferred to nitrocellulose membranes and probed for iGR. Binding of the antibody to the protein on the membrane was visualized by a Western blot chemiluminescence reagent and analyzed by densitometry (Bio-Rad). Antibody specificity for iGR staining was confirmed by preabsorption of the primary antibody with $10\mu\text{g}$ of the

protein sequence used to create the antibody (Cat# PEP-001) and identification of specific and non-specific banding patterns. A diagram of this peptide sequence and proposed structure of the human glucocorticoid receptor is shown in Figure 7.1 (Giguere et al., 1986; Weinberger et al., 1985).

Statistical analysis. Statistical procedures were accomplished using SigmaStat (Jandel Scientific, San Rafael, CA). Developmental changes in measured or calculated variables were assessed using simple linear regression or multiple linear regression. Student's t-test was used to test for differences between the treatment groups and their controls. For the purpose of performing the regression analysis, we assigned a value of (147 + postnatal age) in days to each lamb. Data were expressed as % of control in the steroid-treated fetuses. The null hypothesis was rejected and statistical significance determined when $p < 0.05$.

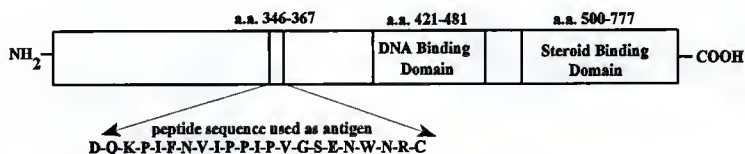


Figure 7.1. Proposed structure of the human glucocorticoid receptor. The peptide sequence from amino acids 346-367 was used as antigen to produce antibody #57 (Cidlowski, et al., 1990), which was used in this study. This peptide sequence was also used in the preabsorption experiments to determine specific staining of iGR. Drawing was adapted from published data (Affinity BioReagents, Neshanic Station, N.J.; Giguere, et al., 1986; Weinberger, et al., 1985).

7.3 Results

The Western blot shown in Figure 7. 2 is representative of the staining observed in these studies. Panel A illustrates specific staining for immunoreactive glucocorticoid receptor protein at approximately 90-94kD and staining of a smaller protein at 46-50 kD. Panel B is a Western blot of the same tissue with the antibody preabsorbed with peptide sequence to which the antibody was raised. Decreased staining intensity in both bands in response to preabsorption demonstrates specificity of staining for the epitope of GR to which the antibody was raised. Protein-antibody binding was determined to be specific by preabsorption of the antibody and a subsequent decrease in staining intensity of protein bands.

In performing the Western blots, the fetal tissue samples were distributed among the gels so that each gel contained a subset of the developmental ages studied. However, statistical analysis of the data was complicated by variation in staining intensity and because of variation in exposure of the film during the chemiluminescence reaction. We corrected for these variations by analyzing the optical density data by multiple regression analysis, using developmental age and gel number as independent variables. This analysis did not detect a significant influence of developmental age on the full-length receptor in either the pituitary or the hypothalamic tissue homogenates (data not shown). The variation attributable to the variations in gel density was statistically significant ($p < 0.05$).

Multiple regression analysis of the 46-50 kD protein in both the pituitary and hypothalamic tissue homogenates revealed a significant influence of developmental age on optical density of the immunoreactive band (data not shown). We further analyzed the results by calculating the ratio of the optical density of the 46-50 kD protein band to the optical density of the 90-94 kD GR band. Analysis of this ratio by linear regression analysis revealed

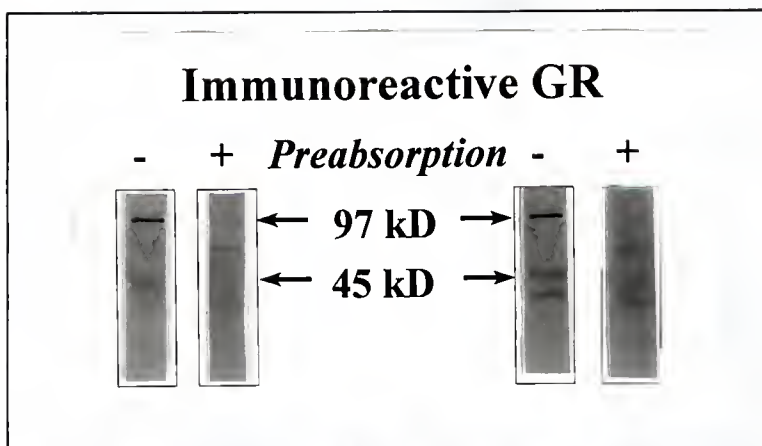


Figure 7.2. Western blot analysis of iGR in pituitary homogenate. Bands specific for iGR epitope are denoted by the arrows.

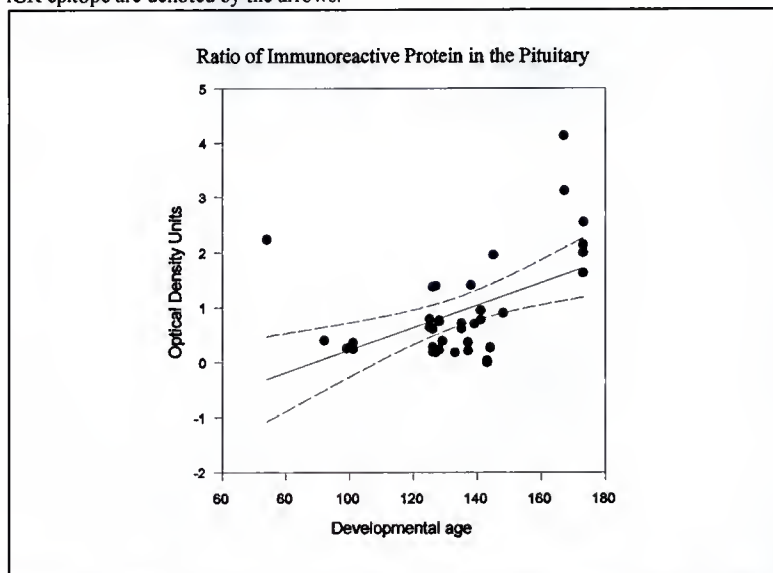


Figure 7.3. Ratio of 46-50 kD iGR protein to the mature iGR protein (90-94kD) in the fetal pituitary as a function of developmental age. Linear regression analysis is significant (solid line, $p < 0.05$). Dashed line represents 95% confidence interval.

a statistically significant increase in optical density as a function of developmental age in both the pituitary (Fig. 7.3) and the hypothalamus (Fig. 7.4). Calculation of this ratio eliminated the need to analyze the data by multiple linear regression analysis, since the procedure normalized image density (data not shown).

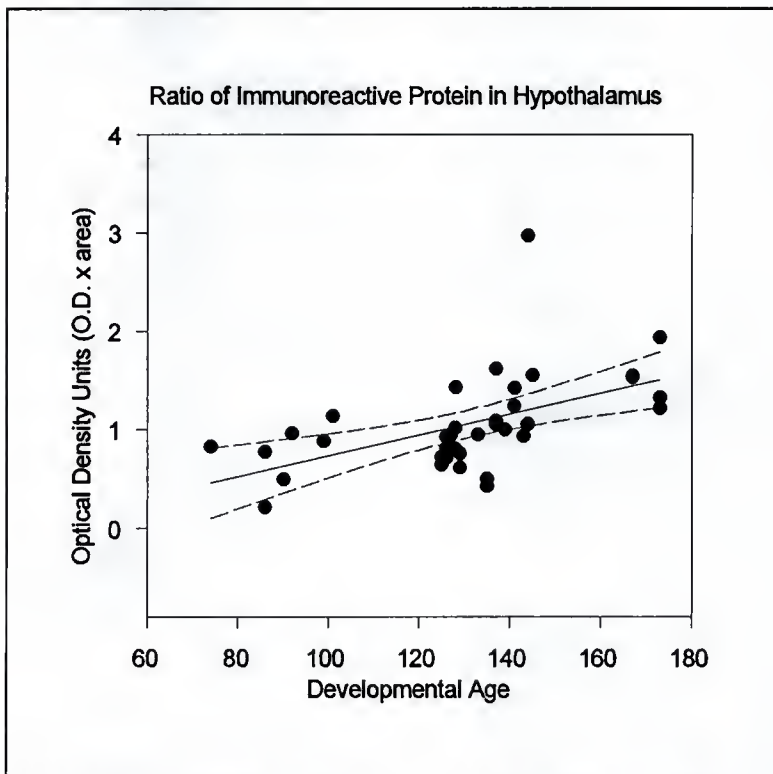


Figure 7.4. Ratio of 46-50 kD iGR protein to mature 90-94 kD iGR protein in fetal hypothalamus as a function of developmental age. Linear regression analysis is significant (solid line, $p < 0.05$). Dashed line represents 95% confidence interval.

iGR protein staining in the steroid-treated fetuses is shown in Table 1. None of the steroid treatments produced statistically significant changes in the ratio of the staining in the 46kD to the 90-94 kD bands.

Table 1: Effect of estradiol, androstenedione, or tamoxifen treatment on the concentration of the ratio of the 90-94kD GR to the 46-50 kD immunoreactive protein in fetal hypothalamus and pituitary.

Treatment	Pituitary (46-50kD/90-94kD ratio)	Hypothalamus (46-50kD/90-94kD ratio)
Estradiol	0.809±0.222 (n=4)	1.040±0.143 (n=4)
Androstenedione	0.504±0.089 (n=8)	0.830±0.209 (n=7)
Tamoxifen	0.659±0.137 (n=4)	0.798±0.078 (n=5)
Control (129±1 days)	0.578±0.111 (n=14)	0.852±0.059 (n=14)
Control (140±1 days)	0.634±0.157 (n=13)	1.240±0.189 (n=13)

7.4 Discussion

In this study, we did not detect any significant influence of developmental age on the abundance of the 90-94 immunoreactive glucocorticoid receptor protein in fetal hypothalamus or pituitary over the ages studied. Unexpectedly, however, we did find evidence for an 46-50kD protein recognized by the GR antibody, whose abundance is significantly changed as a function of fetal and neonatal development in both pituitary and hypothalamus. We suspect that this 46-50kD protein is a proteolytic fragment of the native receptor protein and that increases in this protein may reflect increased rates of intracellular receptor clearance or turnover.

The existence of this 46-50kD protein has been reported by other investigators in *in vitro* preparations. Using an antibody which was different from the one in this study, a

minor band at approximately 45 kD was detected from a cellular preparation (Srinivasan and Thompson, 1990). The authors suggested that it was a proteolytic fragment of the native glucocorticoid receptor. The present study is the first to demonstrate this fragment in an *in vivo* system, and the first to demonstrate that changes in the cellular concentration of the fragment are a feature of normal development. Yang et al., reported that the levels of GR mRNA in fetal hypothalamic and pituitary tissues were relatively constant throughout gestation (between 60 days and term, approximately 145 days) but increased significantly in newborn lambs (Yang et al., 1991). This pattern is similar to the increase that we have demonstrated for the half-length GR protein. The regression analysis demonstrated a significant increase in the 46-50kD protein with development; however, in the pituitary it is evident that the largest increase in this protein occurred in the lambs (Figure 7.3). The change in the hypothalamus appears to be more gradual (Figure 7.4). Nevertheless, it is possible that immunodetection of the 46-50kD protein reflects the increased turnover of the receptor and that there is increased turnover of the GR as a function of age.

The number of GR binding sites in these tissues are relatively constant during gestation (between 70 days and term) and the neonatal period but decrease significantly in adult animals (Rose et al., 1985). Rose et al. (1985) also found that the number of binding sites in the pituitary were approximately 10 fold higher than in the hypothalamus. Binding studies of GR for the purpose of estimating receptor number are complicated by the process of receptor occupancy and activation (Evans, 1988). Accurate determination of glucocorticoid receptor number in binding experiments requires the removal of endogenous steroids by adrenalectomy (Reul and DeKloet, 1985). In the fetus, this

experiment would require adrenalectomy of both mother and fetus. Although we did not compare iGR staining in pituitary to that in hypothalamus (we arranged the samples in the gels to test for changes related to gestational age), our results are consistent with those by Rose, et al. (1985). The abundance of 90-94kD iGR protein in fetal hypothalamus and pituitary was relatively constant throughout gestation.

The antiserum that we used in the present study binds to amino acids 346-367 of the human GR (Cidlowski et al., 1990). This peptide sequence is conserved in the sheep GR (i.e., it is not found in the estrogen, androgen, mineralocorticoid, or progesterone receptor) (Evans 1988). The ability of the antibody used in this study to reveal the half-length GR protein was reproduced with another commercially-available antiserum (#PA1-510) from Affinity Bioreagents). The epitope of this antiserum is also presumably in the amino-terminal end of the GR. The use of these and other antisera for immunocytochemical detection of GR is a technique which has been used by several groups (Antakly and Eisen, 1984; Brink et al., 1992; Wikstrom et al., 1987; Cidlowski et al., 1990; McGimsey et al., 1991). Because immunocytochemistry does not discriminate on the basis of molecular weight, it is possible that some of the iGR identified with this technique actually represents the 46-50kD protein with the same epitope to which the antibody was raised.

We conclude that fetal hypothalamus and pituitary contain molecular forms of iGR which are consistent with the native (90-94kD) GR and an immunoreactive protein, approximately 46-50kD which may represent a proteolytic fragment of the native GR. We could not identify a statistically significant change in the concentration of 90-94kD iGR as a function of development, but we did identify an increase in the concentration of the 46-

50kD protein relative to 90-94kD iGR in both hypothalamus and pituitary. In summary, we did not find a significant difference in the 90-94kD iGR protein over the ages studied in fetal pituitary and hypothalamic tissue homogenates. We speculate that the 46-50kD protein recognized by the antibody is the result of receptor degradation resulting from receptor turnover or down regulation.

CHAPTER 8

EXOGENOUS ESTRADIOL AND ANDROSTENEDIONE ADMINISTRATION SHORTENS GESTATION LENGTH IN THE OVINE FETUS

8.1 Introduction

Parturition in sheep is under control of the fetal hypothalamus-pituitary-adrenal (HPA) axis (Challis and Brooks, 1989; Liggins et al., 1973). Near the end of term (gestation length being approximately 147 days in the uninstrumented animal), there is an increase in drive to the fetal hypothalamus and fetal plasma ACTH secretion increases, resulting in an increase in fetal plasma cortisol concentrations. The increases in plasma ACTH and cortisol concentrations at the end of gestation represent a combination of increased activity of the HPA axis and a decreased sensitivity of the axis to negative feedback inhibition of plasma ACTH secretion by cortisol (Wood, 1988). Cortisol acts at the placenta to increase the activity of an enzyme, cytochrome P450 (17-hydroxylase and 17,20 lyase activities) which increases androgen and estrogen production (Anderson et al., 1975; Pomerantz and Nalbandov, 1975; Steele et al., 1976; Yu et al., 1983).

Estrogen is known to be important for uterine contractility and the initiation of labor and delivery (Liggins, 1974). 17β -estradiol has been shown to increase the activity of the HPA axis in rats and sheep. In a recent study (Saoud and Wood, 1995), we have found that estrogen augments fetal plasma ACTH secretion in response to stress. This effect of estrogens has been seen in adult animals as well (Viau and Meaney, 1991).

In the same study, we also examined the effect of androgens on the fetal HPA axis, as androgens also increase at the end of gestation. Studies in male rats have shown that removal of endogenous androgens, via orchidectomy, resulted in increased plasma ACTH secretion (Handa et al., 1994). This suggests an inhibitory role of androgens in ACTH secretion. In our studies (Saoud and Wood, 1995), exogenous administration of androstenedione to intact fetuses did not alter plasma ACTH secretion but decreased the negative feedback efficacy of cortisol on plasma ACTH secretion. We hypothesize that the increase in fetal plasma estrogen and androgen concentrations that occur near the end of gestation augment the increase in HPA axis activity prior to parturition. Therefore, by prematurely increasing fetal plasma 17β -estradiol and androstenedione concentrations, the time of parturition will also be prematurely advanced. In the present set of experiments, we will examine not only the effect of estrogen and androgen on the timing of parturition, but also the pattern of plasma ACTH and cortisol secretion in these fetuses.

8.2 Materials and Methods

Twenty pregnant ewes of known gestational ages were studied. Eighteen ewes had single pregnancies and two ewes carried twins. Aseptic surgery was performed for chronic implantation of vascular and amniotic fluid catheters and steroid pellets at least 5 days before experimentation.

Fetal surgery. For 24 hours before surgery, the ewes were allowed free access to water but were not permitted food. During surgery, the ewes and fetuses were anesthetized with halothane or isoflurane (0.5 - 3.0% v/v) in oxygen. Fetal arterial and venous catheters and an amniotic fluid catheter were placed as previously described. At

the time of catheter placement, steroid pellets were implanted in the area of the gluteus medius of the fetus. Five fetuses received 5 mg, 21 day 17 β -estradiol pellets, six fetuses received 15 mg, 60 day androstenedione and 17 β -estradiol pellets, five fetuses received 30 mg, 60 day 17 β -estradiol pellets, and 6 fetuses were controls (two fetuses received 30 mg of placebo, two fetuses received 15 mg of placebo and 2 fetuses were sham-controls). In 2 ewes with twins, in one set, one fetus received a 5 mg 17 β -estradiol pellet and the other was a sham control and in the other set, only one fetus was surgically prepared as a sham control. In all cases, the approximate release of the pellets based on the steroid content of the pellets was 240-250 μ g/day. Each fetus received 500 mg ampicillin via the amniotic fluid. The ewes received 750 mg ampicillin IM post-operatively BID for 5 days.

Fetal blood collection and handling. Fetal blood samples were taken every 1-3 days beginning 5 days post-operatively as previously described. Each day the catheters were opened, the fetus was treated with 750 mg ampicillin to the amniotic fluid space via the amniotic catheter. Blood samples were kept on ice until returning to the lab. Samples were centrifuged at 3000 x g for 20 minutes at 4°C in a refrigerated centrifuge (Sorvall RT6000B, Dupont, Newtown, CT). After centrifugation, the plasma was transferred to a clean tube and stored at -20°C until hormones were assayed.

Plasma hormone concentration determination. Fetal plasma ACTH and cortisol concentrations were determined by radioimmunoassay (RIA) as previously described. Fetal plasma androstenedione concentrations were determined by RIA using a kit purchased from Diagnostic Products Co. (Los Angeles, CA) after extraction with ethyl acetate:hexane (3:2). Fetal plasma estrogen concentrations were determined by RIA using an antibody developed by Dr. Kalra's lab after extraction in ethyl ether. The cross-

reactivity of this antibody with estrone is approximately 10% (unpublished observations). Plasma estradiol concentrations were determined after separation by Sephadex LH 20 columns (Isolab, Inc., Akron, OH). Fetal plasma estradiol concentrations were determined at a single time point in all the animals studied (approximately 10 days following surgery).

Statistical analysis. Overall differences between group means were determined by ANOVA and individual differences were determined by multiple comparison tests (Student-Newman-Keuls). Data was expressed as mean $\pm$ SEM. Differences over time in the hormone data was determined by one-way ANOVA. Differences between treatment groups for plasma ACTH, cortisol, estrogen, and androstenedione hormone concentration data was analyzed by regression analysis and data was expressed as single data points. A significance level of $p < 0.05$ was used to reject the null hypothesis.

8.3 Results

Table 8.1 summarizes the fetal blood gases and pHs during the sampling period. The fetuses were all born healthy except for one estradiol and androstenedione treated fetus. This fetus had consistently low arterial PO_2 values. The lungs were partially inflated at necropsy, indicating that the fetus was probably alive at the time of delivery and died shortly after birth. In the 30mg E2 group, complete hormonal data was not available due to catheter failure. However, delivery dates were collected in all 5 fetuses in this group. The hematocrits in the 30 mg E2 fetuses were significantly greater in these fetuses compared to the sham-controls.

Table 8.1. Summary of fetal blood gases, pH, and hematocrit measured during the study.

	pH	P _i O ₂ (mmHg)	P _i CO ₂ (mmHg)	hematocrit
Controls (n=6)	7.362 ± 0.004	18.02 ± 0.31	50.21 ± 0.62	32.6 ± 0.78
5 mg E2 (n=5)	7.334 ± 0.009	23.32 ± 0.72	44.21 ± 0.84	N.D.
15 mg A+E(n=6)	7.344 ± 0.006	17.74 ± 0.44	55.73 ± 0.60	34.4 ± 0.70
30 mg E2 (n=5)	7.362 ± 0.006	17.04 ± 0.37	52.97 ± 1.49	36.8 ± 0.42

The 5 mg E2 fetuses had significantly different values in all variables measured because measurements were made using a different blood gas analyzer than the other 3 groups. However, all variables in all the groups fall within a normal range for the ovine fetus. Data are expressed as mean ± SEM. (N.D. = not determined). The arterial pH of the 30 mg E2 fetuses was significantly greater than the 15 mg A+E fetuses ($P < 0.05$). The arterial PCO₂ of the 15 mg A+E fetuses was significantly greater than the 30 mg E2- and sham-fetuses ($P < 0.05$). This is most likely an artifact in the study since the PCO₂ electrode was replaced in the middle of the study after measurement in the 15 mg A+E fetuses and before measurement in the 30 mg E2- and sham-fetuses. The 30 mg E2 fetuses has significantly greater hematocrits than the 15 mg A+E- and sham-fetuses ($P < 0.05$).

Table 8.2 summarizes the fetal plasma 17 β -estradiol and androstenedione concentrations in fetal sheep approximately 10 days after surgery and the mean gestation length following steroid implantation.. Plasma estradiol concentrations were significantly increased to 26.2 ± 4.0 pg/mg in the 15 mg A+E and 27.4 ± 6.0 pg/ml in the 30 mg E2-implanted fetuses compared to 2.8 ± 1.2 pg/ml in the sham fetuses. Plasma androstenedione concentrations were significantly increased to 343.2 ± 24.2 ng/ml in the 15 mg A+E implanted fetuses compared to 256.4 ± 30.1 ng/ml in the sham fetuses.

Comparison of the mean length of gestation by ANOVA revealed a significant influence of treatment on the day of birth ($F=9.07, df=21, p=0.0007$). Multiple comparison by Student-Newman-Keuls revealed a significantly shorter mean gestation length in the 15 mg A+E implanted fetuses (141.7 ± 0.8 days) compared to the 5 mg E2-treated (144.4 ± 0.7 days), 30 mg E2-treated (147 ± 1.0 days) or the sham-control fetuses (145.8 ± 0.7 days). All other comparisons were not significant.

Table 8.2. Summary of fetal plasma 17β -estradiol and androstenedione concentrations approximately 10 days post-implantation. * denotes significance from control.

	17β -estradiol (pg/ml)	androstenedione (pg/ml)	length of gestation
Control	2.82 ± 1.2	256.4 ± 30.1	145.8 ± 0.7 days
5 mg E2	17.8 ± 0.6 *	N.D.	144.4 ± 0.7 days
15 mg A+E	26.2 ± 4.0 *	343.2 ± 24.2 *	141.7 ± 0.8 days *
30 mg E2	27.4 ± 6.0 *	220.8 ± 15.1	147.0 ± 1.0 day

Fetal plasma ACTH and cortisol concentrations increased significantly over the time periods studied until parturition in the control, 5 mg E2, 15 mg A+E, and 30 mg E2-treated fetuses (Figure 8.1). Data are expressed in terms of gestational age (top panel) and days before parturition (bottom panel). The data appear to correlate better in terms of days before parturition. There was no significant difference in plasma ACTH and cortisol concentrations between the 15 mg A+E and the sham control fetuses. However, the plasma ACTH concentration of the 30 mg E2 and the 5 mg E2-treated fetuses was significantly greater compared to the other 2 groups.

The plasma cortisol concentrations are diagramed in Figure 8.2. Data are expressed in terms of gestational age (top panel) and days before parturition (bottom panel). Plasma cortisol concentrations increased significantly in all 4 experimental groups.

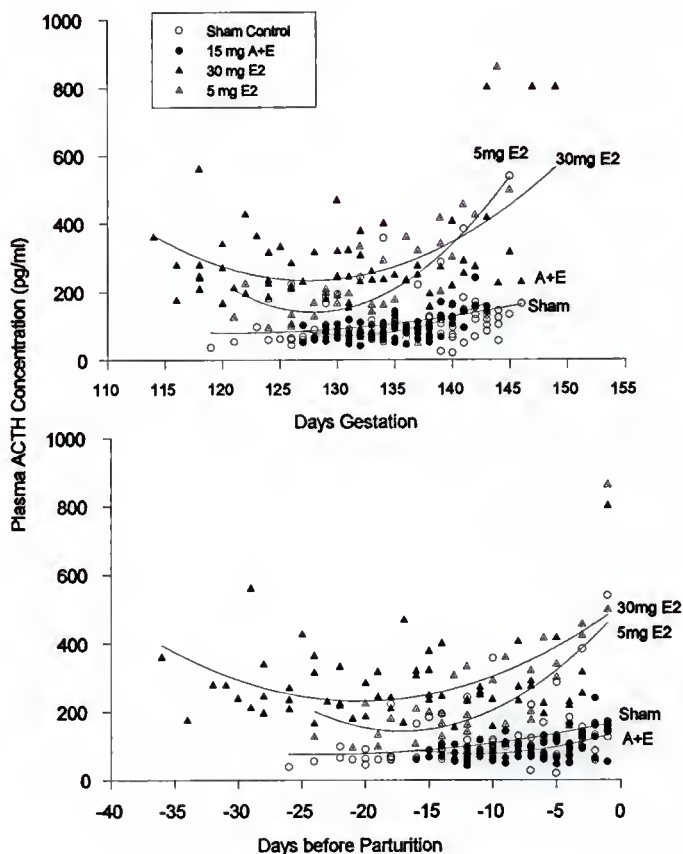
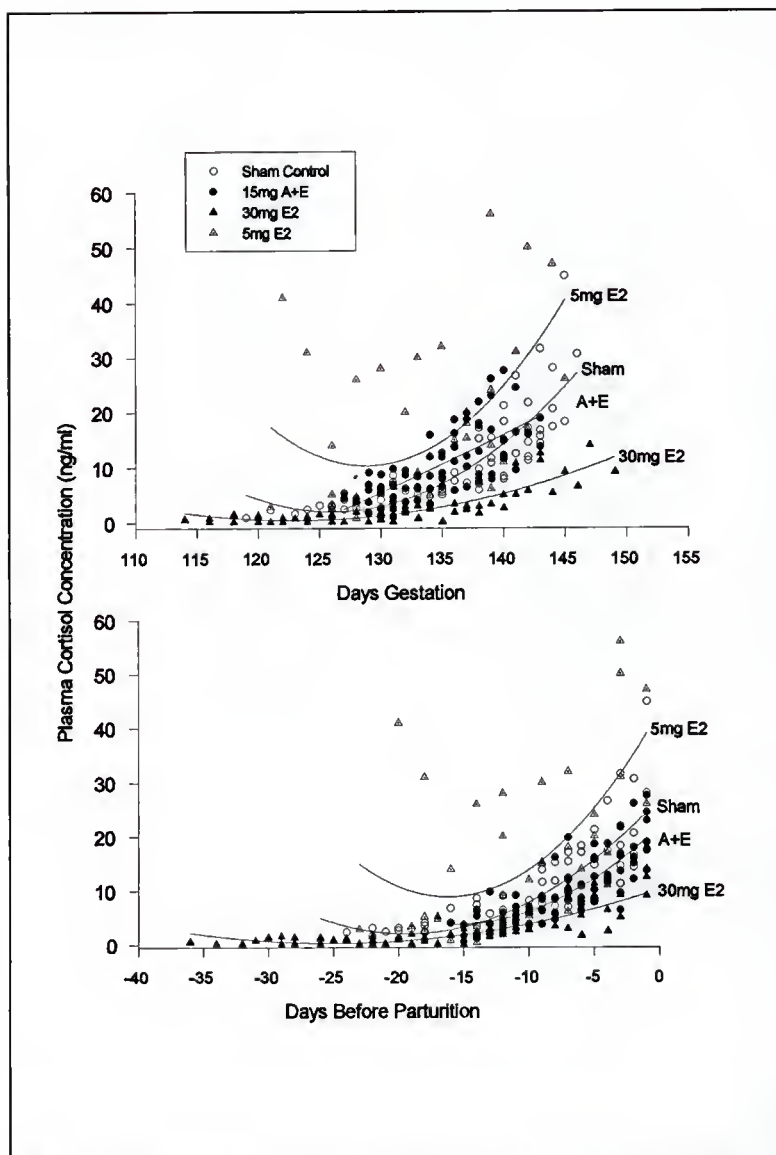


Figure 8.1. Fetal plasma ACTH concentrations versus days gestation (top panel) and days before parturition (bottom panel). Lines represent 2nd order regressions.



As with the plasma ACTH concentrations, there was no significant difference between the 15 mg A+E and the sham control fetuses. However, the plasma cortisol concentrations in the 30 mg E2-treated fetuses were significantly lower than the other 3 groups and 5 mg E2-treated fetuses were significantly higher than the other 3 groups.

8.4 Discussion

With the administration of 15 mg androstenedione and 15 mg 17 β -estradiol (each, approximately 250 μ g/day), we were able to significantly shorten the length of gestation in sheep. In previous work, we have shown individually that estradiol augments fetal plasma ACTH secretion and that androstenedione decreases the efficacy of cortisol negative feedback on fetal plasma ACTH secretion (Chapter 4). It has long been known that increases in fetal plasma ACTH and cortisol secretion at the end of gestation trigger parturition in this species (Challis and Brooks, 1989; Liggins et al., 1973). These increases are a result of increased drive to the HPA axis and a decrease in cortisol negative feedback efficacy (Wood, 1988). We propose that increased estrogen and androgen from the placenta are an important component in this process. These results suggest that gonadal steroids from the placenta play an important role in the control of parturition in this species.

Many studies have been performed in which ACTH or cortisol was infused into the fetal sheep and parturition induced (Liggins, 1968; Liggins, 1969; Kendall et al., 1977; Thomas et al., 1978; Lye et al., 1983). To date, this is the first study in which a substance that occurs endogenously was infused and plasma ACTH and cortisol concentrations were increased, resulting in premature delivery. The plasma concentrations of estradiol and

androstenedione obtained in the treated fetuses were significant, but they were also within the normal physiological range. This is very important in deciding the relevance of this finding. The plasma concentrations we observed in the treated fetuses were similar to concentrations seen in fetuses at a later gestational age.

Parturition was induced prematurely in only the androstenedione/estradiol-treated fetuses. In the two estradiol treatment groups, the length of gestation was not significantly different from the sham-control fetuses. However, plasma ACTH and cortisol concentrations in the 30mg estradiol-treated fetuses were somewhat curious. Although the plasma ACTH concentrations in this group were significantly greater than the control fetuses, the plasma cortisol concentrations were significantly lower. It appears that although secretion of ACTH from the pituitary gland was stimulated, the adrenal gland in these fetuses was unresponsive to ACTH. It has been shown that high molecular weight forms of ACTH can inhibit cortisol secretion from the adrenal in the fetus (Jones and Roebuck, 1980; Roebuck and Jones, 1980). It is possible that this is what might be occurring in this treatment group, although we have not determined the molecular weight profile of the plasma ACTH in these fetuses.

The variability in plasma ACTH values were visually apparent (Fig. 8.1). The reasons for this variability are many. To obtain a true pattern of plasma ACTH secretion, the sampling frequency should be much greater due to the pulsatile nature of secretion from the pituitary. These experiments have been performed successfully and there is much data in the literature to support the exponential increase of plasma ACTH and cortisol concentrations that occur prior to parturition (Magyar et al., 1980; Jones et al., 1977; Norman et al., 1988). However, frequent sampling can stimulate plasma ACTH secretion.

Because the nature of the experiments was to determine whether gonadal steroids could induce premature parturition, we were interested in obtaining an index of the change in plasma ACTH and cortisol concentrations. In order to reduce the variation in sampling from day to day, the timing of sampling was kept constant within each experimental group.

As was seen in the estradiol-treated experimental groups, the administration of exogenous estradiol was not sufficient to induce premature parturition. ACTH was significantly increased in these fetuses, but cortisol secretion was not suggesting an inability of the adrenal gland to respond to the pituitary ACTH. From previous work, we know that full activation of the fetal HPA axis is necessary for parturition. In adrenalectomized fetuses, although fetal plasma ACTH secretion increases because of loss of glucocorticoid negative feedback, parturition is delayed (Dost and Holm, 1968). In our studies, parturition was induced in only the androstenedione- and estradiol-treated fetuses. The combination of increased pituitary ACTH secretion (action of estradiol) and the decrease in cortisol negative feedback efficacy (action of androstenedione) appeared to stimulate the activity of the fetal HPA axis and induce parturition.

In conclusion, the administration of exogenous estradiol and androstenedione to late gestation fetal sheep induced parturition prematurely. The administration of estradiol alone was not sufficient to produce this effect. We believe the independent actions of each steroid (increased ACTH secretion and decreased cortisol negative feedback sensitivity) mimic what occurs in the normal process of parturition. We speculate that estrogens and androgens are an important component in the induction of parturition in the sheep.

CHAPTER 9

SUMMARY

Preterm labor is still a major concern in this day and age. Despite efforts to inform the public about proper nutrition and the need to seek prenatal care, a significant number of infants are being born prematurely. Approximately 5-10% of all deliveries in North America and the United Kingdom occur prematurely, before 37 weeks of gestation. This figure can be higher or lower depending on the developmental status of the country of origin and the socio-economic status of the mother. Complications due to premature delivery (and immaturity of the neonate) contribute significantly to the life or death of these infants (Creasy, 1980; Hall, 1985).

A number of different factors can predispose a mother to preterm birth. Among these are socio-economic factors, maternal age, habits of the mother (including drinking, smoking and drug abuse), and type of work environment. Additional medical factors include sepsis, chronic infection, stress, surgery, and multiple fetuses (Challis and Olson, 1988). Women who have had a preterm birth have a higher incidence of preterm births with each subsequent pregnancy (Hall, 1985). However, these factors only contribute to 50% of all preterm deliveries.

The question that remains is what factors determine preterm delivery in the remaining population of mothers who experience this problem? It is the idiopathic preterm deliveries that perplexes the medical community most. In order to understand the reasons for premature delivery, we must first understand the mechanisms of normal birth.

The purpose of the preceding experiments was to understand some aspects of parturition. We used sheep as a model in these experiments for several reasons. The body of literature involving the ovine fetus is appreciable. This species is widely accepted in the research community as a model for parturition and results involving the sheep can be evaluated as relative to parturition in primates. One major reason for using this species is its size and domesticity. The fetus is relatively large (compared to rats) and similar in size to human fetuses. Surgical manipulations, such as placement of indwelling catheters, removal of endocrine organs, placement of electrodes, etc. can be performed with ease. Because of the size of the fetus, fairly large blood samples can be drawn for endocrine measurements with little adverse effect to the fetus.

Experiments in non-human primates are limited by the sensitivity of the uterus to surgical manipulation. In sheep due to quiescence of the uterus, the fetus can be surgically manipulated without threat of spontaneous delivery following surgery. In primates (including humans), the uterus is very sensitive to manipulation and responds with spontaneous activity and contractions which sometimes leads to abortion of the fetus. This is the main reason for using sheep in parturition experiments. Although the two species are not the same, information obtained in one species can be used to understand the other.

In sheep, it is the fetus that ultimately controls the timing of birth. The process of parturition initiation involves the hypothalamus-pituitary-adrenal axis of the fetus directly. Stimulation of any part of this axis will induce premature delivery. Unlike premature deliveries involving trauma or sepsis, fetuses that are induced by ACTH or cortisol secretion usually have well developed lungs and are able to survive on their own (Liggins,

1968). This is indicative of the normal sequence of birth in the sheep. Glucocorticoids are necessary for lung maturation as well as being intricately involved in the process of parturition. The question still remains, what is the key to initiating parturition?

With the experiments performed, we looked at a number of factors involved in parturition. We examined the developmental changes that occur in hypothalamic releasing factors for ACTH. Tissue content of iCRF and iAVP increased significantly with over the ages studied. Only the hypothalamic content of iCRF increased over gestation; the developmental increase in iAVP was only significant if the neonatal period was considered. We believe this suggests activation of the fetal hypothalamus; increasing hypothalamic content of ACTH releasing factors to increase the secretory capacity of the hypothalamus. Further experiments are needed to substantiate this idea. We also found, based on the molecular weight profile of the hypothalamic homogenate, iCRF and iAVP contained processed hormone as well as immunoreactive precursor peptides. Although tissue content of these releasing factors increased, the peptide that was secreted may or may not have been biologically active.

In order to confirm these findings, experiments involving portal plasma sampling in the fetus throughout gestation and measurement of CRF and AVP would be necessary. Does secretion of CRFs increase with gestation? Is this secretion mainly CRF or AVP or does the combination of CRFs determine plasma ACTH secretion? Finally, what is the molecular weight form of the products being secreted from the hypothalamus and does processing increase with gestation? These are all valid questions that may shed light on activation of the fetal hypothalamus.

We also examined the pituitary content of iACTH and the changes that occur with development. Not only did the tissue content of ACTH increase developmentally, but there was also an increase in processing of ACTH₁₋₃₉ from POMC. The increase in pituitary ACTH content would result in increased secretory capacity of the fetal pituitary, which may or may not play a role in increased plasma ACTH secretion at the end of gestation. It is accepted that plasma ACTH concentrations increase near term, however it is not known if this is due to increased frequency of ACTH secretion, increased ACTH secretion with each pulse of secretion, or a combination of the two. As is already known, hormones from the pituitary are secreted in an episodic fashion. If the frequency or the amplitude increase, then the plasma levels will increase. A detailed study of fetal pituitary ACTH secretion should answer this question relatively easily. If frequency is increased, then this is probably due to an increase in frequency of CRFs from the hypothalamus. If amplitude is increased, then more ACTH is released per pulse indicating that pituitary stores are important.

As mentioned above, the processing of ACTH₁₋₃₉ is also increased developmentally. Much work examining the significance has already been done. High molecular weight forms of ACTH inhibit adrenal secretion of cortisol. This may be important in maintaining a low circulating level of glucocorticoids while the fetus is developing. The possible significance of this may be to keep cortisol low so ACTH secretion will continue and be able to influence the maturation of the adrenal without stimulating cortisol secretion which would shut off the ACTH. Since glucocorticoids are stimulatory to parturition, it would be beneficial to maintain concentrations at a low level until the fetus is fully developed. Once this has occurred, a signal may be sent to the brain

and processing of ACTH would be increased, allowing fetal adrenal stimulation to occur. This is, of course, speculation. Understanding the factors involved in the processing of pituitary ACTH is integral in understanding the activation of the HPA axis.

The increase in fetal plasma ACTH and cortisol secretion that occurs at the end of gestation in the fetal sheep is dependent on two events; the first being increased stimulation, or "drive", to the axis and the second being a decrease in cortisol negative feedback sensitivity. The ability of a hormone to influence a cell or tissue is dependent on receptors for that hormone being present. Cortisol exerts its negative feedback effects on the hypothalamus and the pituitary. We postulated that the inability of cortisol to reduce ACTH secretion may be a receptor mediated event. In other words, a decrease in receptor protein might be responsible for the ineffectiveness of cortisol to turn off ACTH secretion. This could be either at the level of the hypothalamus (cortisol influencing the CRFs) or the pituitary.

Using an antibody to the glucocorticoid receptor and Western blotting techniques, we examined the developmental changes in iGR protein in the fetal hypothalamus and pituitary. In these studies, we did not find a change in the native 94kD receptor protein. We did find a novel 50kD protein fragment which bound the GR antibody in both tissues. The density of this band increased significantly with development. The significance of this 50kD protein is unknown at this time. We believe it represents a proteolytic fragment of the receptor and possibly down-regulation of the receptor (or receptor turn-over) as gestation precedes. This would explain the reduction in glucocorticoid negative feedback sensitivity that is seen in the term fetus. Many more experiments are necessary before this can be substantiated, but we speculate that this 50kD protein is indicative of receptor

turnover. Since the 50kD protein increased significantly in both the hypothalamus and pituitary, we suggest, also, that both of these sites are important in the reduction of cortisol negative feedback efficacy.

All of the above experiments used tissues from fetuses and lambs of varying ages, looking at various factors as they occur naturally. In the following experiments, we altered the endocrine environment of the fetus to determine the possible effects of gonadal steroids on plasma ACTH secretion. We decided to test the effects of estrogens and androgens on the HPA axis of the fetus based on previous work in the adult and because plasma concentrations of these hormones increase in the fetus with gestational age. As mentioned earlier, Nathanielsz et al. (1973) stated that the key to parturition was to find a factor that was present in the fetus and was removed with parturition. Placental production of estrogens and androgens fits this scenario. Fetal plasma cortisol concentrations increase and stimulate the production of estrogens and androgens. The role of estrogens in the initiation of labor and delivery is well known. However, a possible role of androgens and estrogens in activation or stimulation of the fetal HPA axis is a new thought.

What is known about the fetal HPA axis is diagramed in Fig. 9.1. Near the end of gestation, stimulation to the hypothalamus is increased and plasma ACTH and cortisol secretion is increased. The axis is also subject to stimulation from various inputs (labeled as stimuli) which can increase plasma concentrations of ACTH and cortisol. Cortisol normally exerts a negative feedback effect on the hypothalamus and pituitary to turn off ACTH secretion. Near term, this feedback becomes ineffective, and plasma ACTH and cortisol concentrations increase in an exponential manner, eventually initiating parturition.

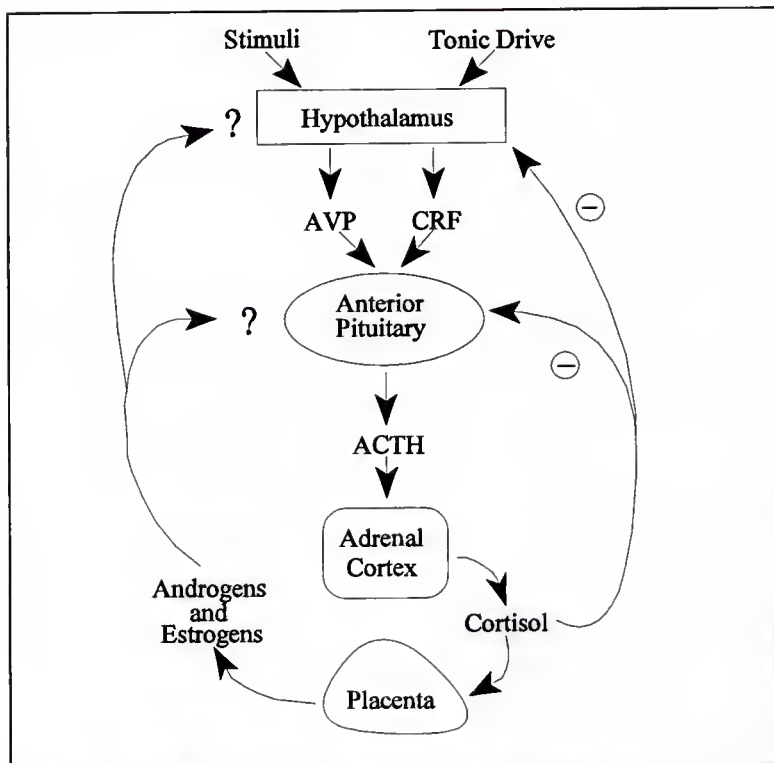


Figure 9.1. Diagram of the fetal hypothalamus-pituitary-adrenal axis.

This is accomplished by cortisol increasing plasma estrogen concentrations by shifting the production of progesterone from the placenta to estrogens.

In the Fig. 9.1, we have depicted the actions of androgens and estrogens on the HPA axis as questions. However, we feel that we may be able to answer these questions, at least in part. In the first experiments, we examined the possibility that estrogens or androgens influenced the HPA axis by effecting the negative feedback regulation of ACTH secretion in the fetus. We designed "feedback experiments" in which the activity of the

fetal HPA axis was measured either unperturbed or after suppression of the axis with a cortisol infusion. The fetal response to stimuli was measured in each circumstance and the plasma ACTH responses were compared. The cortisol infusion was chosen in order to suppress fetal ACTH secretion by approximately 50% in control fetuses. In fetuses treated with estradiol, the activity of the HPA axis was augmented and the efficacy of the cortisol negative feedback inhibition was variable. In other words, estradiol-treated fetuses had significantly greater plasma ACTH concentrations compared to control fetuses, both in the basal condition and after stimulation. Estradiol did not significantly effect the ability of cortisol to suppress plasma ACTH secretion. Therefore, we concluded that estradiol influences the fetal HPA axis by augmenting plasma ACTH secretion and amplifying the response to stimuli, resulting in increased plasma ACTH secretion.

We found a very different result in the androgen-treated fetuses. We implanted fetuses with androstenedione and performed similar feedback experiments as with the estradiol-treated fetuses. In these fetuses, there was no alteration in plasma ACTH secretion following the control infusion. Basal and stimulated plasma ACTH concentrations were not different than the control fetuses. Following the cortisol infusion, the control fetuses had plasma ACTH concentrations that were approximately 60% of those in the control infusion. However, plasma ACTH concentrations in the androstenedione-treated fetuses were not suppressed following the cortisol infusion. The androstenedione had blocked the normal cortisol negative feedback regulation of ACTH secretion. The results in the androstenedione-treated fetuses closely resembles what occurs naturally in the term fetus. We would conclude that increases in exogenous fetal plasma androgen concentrations decrease the efficacy of cortisol negative feedback regulation of plasma ACTH secretion in the fetus.

The final experiment we performed was to test the theory that increases in fetal plasma androgen and estrogen concentrations stimulate the initiation of parturition in the sheep. This is accomplished by estrogens increasing the activity of the HPA axis and androgens decreasing cortisol negative feedback sensitivity, thus allowing for the exponential increase in plasma ACTH and cortisol concentrations that stimulate labor and delivery. In these fetuses, we implanted estradiol and androstenedione pellets (same dose as was used in the previous experiments, 250mg/day) and measured plasma hormone concentrations until the fetuses delivered. In the treated fetuses, the mean gestational age at the time of delivery was 141 days compared to 145 days in the control fetuses, which was statistically significant. We also implanted fetuses with estradiol alone (250mg/day or 500mg/day), whose time of delivery was not significantly different than the controls. In the fetuses with the 500mg/day estradiol, we saw an significant increase in basal ACTH secretion and a significant decrease in plasma cortisol concentrations. This further supports the conclusion of estrogen augmenting plasma ACTH secretion, but raised the question of bioactivity of the ACTH that is secreted from the pituitary.

We can summarize the findings of these experiments as follows (Fig. 9.2): As the fetus develops, the pituitary secretes ACTH which stimulates maturation of the fetal adrenal. Secretion of cortisol from the adrenal gland can act on the hypothalamus and pituitary gland to turn off ACTH secretion. Cortisol can also act on the placenta to increase androgen and estrogen production. Estrogen acts at the hypothalamus and/or the pituitary to augment plasma ACTH secretion, basally or in response to stimuli. Androgen acts at the hypothalamus and/or the pituitary to inhibit glucocorticoid negative feedback regulation of plasma ACTH secretion. As the adrenal secretion of cortisol increases, so

does the production of androgens and estrogens. Eventually, a positive feedback loop occurs, in which plasma ACTH and cortisol concentrations increase exponentially, and labor and delivery are initiated.

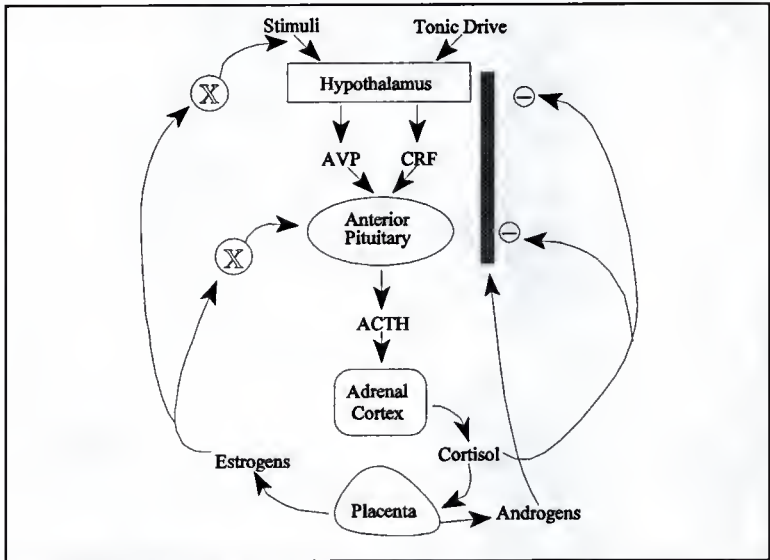


Figure 9.2. Diagram of the fetal HPA axis. Proposed actions of androgens and estrogens on the axis. "X" denotes a multiplier action or augmentation and the solid bar represents an inhibition of cortisol negative feedback.

We have proposed many possibilities for the roles of androgens and estrogens in the initiation of parturition in sheep. These are but a few of the experiments needed to prove conclusively that these gonadal steroids are intimately involved in this process. We did not intend to prove that these steroids were the key to parturition, only that they are another piece of a larger puzzle. The action of androstenedione on cortisol negative feedback efficacy should prove to be an integral part in the initiation of parturition. The

relative significance of estradiol on the stimulation of the HPA axis is still somewhat questionable. One of the biggest questions that remains is what is the signal that increases drive to the HPA axis? Estrogens may fit this function, but their concentrations are increased by cortisol which is increased by activity of the HPA axis. The signal may be purely a maturational one. When the fetal adrenal reaches critical mass, cortisol secretion may then be great enough to increase estrogens and then activate the HPA axis. These are important questions and hopefully experiments performed in the future will elucidate some of the answers.

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BIOGRAPHICAL SKETCH

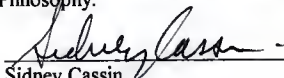
Christine Jean Saoud was born Christine Taranovich in Waterbury, CT, on April 5, 1969. Her family moved to Melbourne, FL, in 1971. Christine attended school in Melbourne and received her high school diploma from Melbourne High School with honors. Following graduation in 1987, Christine was accepted to the University of Florida in Gainesville and enrolled for the 1987 fall term. As an undergraduate, she majored in zoology while working in a research lab under the supervision of Dr. Charles Wood as an assay technician. In the summer of 1991, Christine was awarded a Bachelor of Science. In the fall of 1991, she entered graduate school in the College of Medicine, Department of Physiology in order to pursue the Doctor of Philosophy degree. Christine continued to work with Dr. Charles Wood in the areas of fetal, cardiovascular and reproductive physiology. Following the end of her Ph.D. program in December of 1995, Christine, along with her husband, will relocate to Maryland where they intend to settle down.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



Charles E. Wood, Chair
Professor of Physiology

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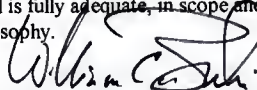
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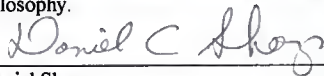
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William Buhi
Professor of Biochemistry and Molecular
Biology

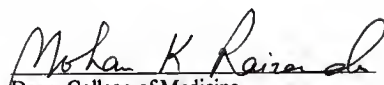
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Daniel Sharp
Professor of Animal Science

This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December 1995


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